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Chromatographic Studies on Nucleic Acids

2. THE QUANTITATIVE ANALYSIS OF RIBONUCLEIC ACIDS

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(*Received* 18 *October* 1949)

The nucleic acids are such universal constituents of biological systems and their function is probably so important that it is not surprising that much labour has been spent on the study of their composition. Until very recently there have been no methods of analysis which could be considered more than semi-quantitative, and it now seems doubtful whether many published analyses are even approximate. While it is now possible to separate and estimate quantitatively mixtures of purine and pyrimidine bases and some of their derivatives by means of paper strip chromatography (Hotchkiss, 1948; Vischer & Chargaff, 1948; Markham & Smith, 1949*b*), it is by no means as simple to convert nucleic acids into these compounds quantitatively. We have investigated many methods for the hydrolysis and estimation of nucleic acids, and have found it impossible to get satisfactory results by any published method. By combining our chromatographic technique with a simple method of quantitative hydrolysis and a solvent system which resolves the hydrolysate, we have evolved a new method of analysis which, in addition to being more sensitive than existing methods, is accurate and simple.

The hydrolysis of ribonucleic acids

Various methods have been used for the hydrolysis of ribonucleic acids. These depend upon the relative lability of the various linkages in these compounds to different treatments. For convenience they may be tabulated as follows: (1) Hydrolysis by NaOH or KOH at low temperature (20–37°), which was employed by Schmidt & Tannhauser (1945), liberates the nucleotides of the pyrimidine and purine bases. (2) Hydrolysis by NH_3 under pressure (Levene & Bass, 1931) is supposed to liberate nucleosides. (3) Hydrolysis by pyridine-water (Bredereck, Martini & Richter, 1941) is also said to liberate nucleosides. (4) Hydrolysis with weak mineral acid at 100°. This liberates the purines and the pyrimidine nucleotides. (5) Hydrolysis with mineral acids at high temperatures. This liberates both purine and pyrimidine bases. (6) Hydrolysis with formic acid at 175° (Vischer & Chargaff, 1948). (7) Enzymic methods. We have not investigated these.

Method (1) has been used, apparently with success, by Chargaff, Magasanik, Doniger & Vischer (1949). The four nucleotides were not resolved on one chromatogram, so the solution was fractionated or a mixture of two nucleotides estimated from the absorption of the mixture at two wavelengths followed by the application of simultaneous equations. Using Whatman no. 1 paper and several mixtures designed to simulate as closely as possible the conditions described by these authors (*iso*butyric acid saturated with water, in the presence of NH_3 vapour), we were unable to obtain a satisfactory separation of an artificial mixture of uridylic and cytidylic acids.

Method (2) was used extensively by Levene and his colleagues. The actual temperature used was 145°, but as they invariably gave the temperature of the autoclave oil bath, it is commonly believed that a temperature of 175° was used for this hydrolysis (Levene & Jacobs, 1916). A certain proportion of the hydrolysate consists of nucleosides, but the method is not suitable for quantitative use as deamination takes place and some free bases are formed. For instance, adenosine treated by this method is partly converted to adenine, hypoxanthine and inosine.

Method (3), which has been used by Reichart (1948), involves refluxing with pyridine-water for 4·5 days and, besides being tedious, is not quantitative. Chromatograms of such a hydrolysate showed the presence of at least seven substances.

Method (4), hydrolysis with mineral acid, usually N-HCl at 100° for 1 hr., liberates the purine bases quantitatively. The pyrimidine nucleotides are also liberated, together with negligible amounts of the pyrimidine nucleosides. This is the method which was adopted and will be discussed at length later.

Method (5), hydrolysis with mineral acids at temperatures above 100°, liberates the purines and also free pyrimidines and some pyrimidine nucleosides. The purines are deaminated, and are eventually converted to glycine, formic acid, NH_3 and CO_2 (Krüger, 1892; Wulff, 1893). The cytosine is deaminated to uracil.

Hydrolysis with formic acid at 175° (method 6) liberates the bases with some loss of the purines and deamination. The cytosine is not deaminated to any extent, but for some reason the method does not give consistent or quantitative results for the pyrimidines, especially uracil. We have made many hydrolyses with formic acid for various periods of time in an unsuccessful attempt to make the method suitable for use with ribonucleic acids. It is an excellent method for the hydrolysis of uridine to uracil, giving quantitative recovery of uracil. In addition uracil added to thymus deoxyribose nucleic acid has been recovered quantitatively after hydrolysis.

EXPERIMENTAL

Chromatographic methods

Source of ultraviolet light. All chromatograms were run on Whatman no. 1 paper. The spots on the developed chromatograms were located in ultraviolet light (Markham & Smith, 1949*a*, *b*). Recently we have used as a source of ultraviolet light a Mazda 80 W. MB/V mercury lamp (British Thompson Houston) with the outer glass envelope removed. This lamp and the choke for controlling it are cheap. The lamp provides an intense source of ultraviolet light, which, though not as rich relatively in short-wave ultraviolet light as lower pressure mercury lamps, has a higher absolute intensity, thus allowing much shorter exposures and illuminating evenly a much greater area (an area of 12 × 14 in. of chromatogram being exposed for 15 sec. at a distance of 5 ft.). The filtered light from this lamp gives radiation predominantly of wavelength 265 mμ., rather less of wavelength 253·7 mμ. and a certain amount of general background.

Several people employing our method have used low-pressure mercury arc lamps (which emit mainly at 253·7 mμ.) without filters. While these give useful results, the definition is not as good as that obtained with a high-pressure arc used with filters.

Solvents. Having investigated the various hydrolysis procedures mentioned in the introduction we decided upon method (4), hydrolysis with N-HCl at 100°. The time chosen was 1 hr. as maximal yields of purines are given in this time. During the early stage of the work we employed as a routine a solvent containing 77% *n*-butanol, 13% water and 10% formic acid (v/v/v) (Markham & Smith, 1949*b*), which moves the purines, but leaves the nucleotides at the starting spot. It was desirable, however, to have a solvent which would move the nucleotides as well. From previous experience it was known that solvents containing more water than water-saturated *n*-butanol made the nucleotides move; on the other hand, it was essential to use an acidic solvent, as acid, besides accelerating the movement of the nucleotides, makes it possible to use relatively large quantities of guanine which is rather insoluble in neutral solutions or in the presence of NH_3. Experiments with formic acid–propanol-water mixtures showed that the pyrimidine and purine nucleotides moved and increased in R_F with increasing acid concentration and also with increasing water content. *sec.*-Butanol-water mixtures were very similar, a mixture of 80% *sec.*-butanol, 20% water and 10% formic acid (v/v/v) proving incidentally a very useful solvent mixture for purines and pyrimidines. It was not possible, however, to get complete resolution in one dimension with formic acid mixtures, though this is easily possible in two dimensions. We therefore investigated other acids, both stronger and weaker than formic acid. HCl proved most suitable, and, eventually, by adjusting the water content and acidity of the solvent system, we arrived at a mixture consisting of 0·8N-HCl made up in a solution containing 70% by volume of *tert.*-butanol, the remainder of the mixture being water. To make this solution, take 700 ml. of pure *tert.*-butanol (at 26°), add 132 ml. of constant boiling HCl and make up to 1 l. at 20°. This solvent has several advantages. It separates guanine, adenine, cytidylic acid and uridylic acid in that order on a short strip of paper (Fig. 1), the R_F values being approximately 0·21, 0·32, 0·48 and 0·79 respectively, adequate resolution being given with a total length of flow of 25 cm. The HCl front moves with the butanol front (in weak HCl solutions it lags behind) and the opaque brown line which coincides with the former lies quite clear of the uridylic acid spots. Being a very acidic solvent it is not necessary to neutralize the hydrolysate, the spots being put directly on to the papers, and in addition all the spots, including the guanine spot, are compact and show no tendency to tail. The only disadvantage of this solvent is that it runs rather slowly, a single run taking just under 2 days in battery-jar tanks (14 × 10 × 35 cm. high, internal dimensions) at 18–20°. It is necessary to dry the developed chromatograms carefully in a current of warm air without exposing them to high temperatures, which would cause the acid to attack the paper. Strips are hung with clothes pegs on a line, their lower ends being 20 cm. above a low-temperature 1 kW. electric radiator in a fume cupboard with the ventilating fan running. Drying takes 0·5–1 hr.

This solvent system is only suitable for the process for which it has been designed. It is not able to resolve the four nucleotides from yeast nucleic acid on a short strip of paper, and being very acid, is not recommended for use with labile substances; e.g. it hydrolyses yeast nucleic acid to nucleotides on the paper itself. Another point to note is that small changes in either the HCl or the water content of this solvent change the R_F values of the four substances in the hydrolysate and may make the resolution worse. On the other hand, the relative positions of these substances remain the same, even if the solvent composition varies over quite a large range. Generally it is unnecessary to run marker substances because of this, and, in any case, the absorption spectra of the substances in the spots are characteristic. It is also possible in case of doubt to elute a spot, evaporate it to dryness and subject it to further treatment. Cytidylic acid, for example, may be hydrolysed in formic acid by the method of Vischer & Chargaff (1948) and then be identified by running as a spot in *n*-butanol-formic acid or some other suitable solvent system.

Analytical methods

Phosphorus. After incineration of the sample with perchloric acid P was estimated as strychnine phosphomolybdate by Tisdall's (1922) colorimetric method.

Ribose. Purine-bound ribose was estimated by Militzer's (1946) orcinol method, as modified by Markham & Smith (1949*c*), using xylose as standard. Tests with the various ribosides showed that this modified method gives quantitative values for purine-nucleotide sugar, and does not give appreciable colour with that bound to pyrimidines.

Nitrogen. This was estimated by the usual micro-Kjeldahl method (Markham, 1942).

Biuret reaction. This was carried out using a drop of 0·5% $CuSO_4$ and KOH pellets in excess. Under these conditions a small quantity of ethanol added to the solution is salted out and takes all the biuret colour with it. Consequently the method is many times more sensitive than the usual one. (This technique, though discovered many years ago, does not seem to be at all well known. We have not been able to find the original reference.)

Hydrolysis of nucleic acid and estimation of purines and pyrimidine nucleotides

The nucleic acid was either evaporated to dryness in a current of air in a small evaporating tube immersed in a water bath at 100° or precipitated by dilute HCl and spun

down to the bottom of a small tube. N-HCl was then added to give a final concentration of the nucleic acid of about 10–20 mg./ml. The specific volume of nucleic acid was taken as about 0·6, the amount of N-HCl chosen accordingly; and 100 μl. of acid were usually quite sufficient. A small glass bead was added for mixing and the tube sealed and heated for 1 hr. at 100° in a water bath. It was cooled, opened, and 10–20 μl. quantities of the hydrolysate were measured out in capillary pipettes and placed on the filter paper about 6 cm. from the top. Two spots were placed on each sheet 9 × 34 cm., with 5 cm. between the spots for the blank strip. The paper was then developed in the *tert.*-butanol-HCl mixture for 2 days, dried and printed photographically. The fluorescence quenching technique of Holiday & Johnson (1949) may also be used, though we think that the spots are more obvious by our method. This is to be expected on account of the high contrast of the photographic materials. We find that guanine fluoresces quite brilliantly when excited by the 253·7 and 260 mμ. light. The fluorescence effects may also be recorded photographically. If a thin sheet of cellulose nitrate is placed between the chromatogram and the reflex document paper, only the fluorescent light is able to reach the latter. The exposures are, of course, much longer. This technique could be used for recording the fluorescence of substances in 365–366 mμ. light if a suitable filter were substituted for the cellulose nitrate film.

The spots on the chromatogram were cut out, together with the corresponding blanks, and each was extracted for at least 18 hr. in 5 ml. of 0·1 N-HCl in a test tube. The densities of the solutions were then read in a Beckman spectrophotometer, the following data being used:

Guanine	$D_{250} = 10{\cdot}6$
Adenine	$D_{260} = 13{\cdot}0$
Cytidylic acid	$D_{280} = 12{\cdot}95$
Uridylic acid	$D_{260} = 9{\cdot}89$

(The density D is for a cell 1 cm. thick, the subscript referring to the wavelength in mμ. The concentration is 10^{-3} M. The values for cytidylic and uridylic acids are derived from the data of Ploeser & Loring (1949).)

Materials

Yeast nucleic acid. A commercial sample of about 2 g. of yeast nucleic acid was dissolved in 10 ml. of water by addition of the minimal amount of NH_3 and then precipitated by addition of 2 vol. of ethanol together with a few drops of N-HCl. The precipitate, collected by centrifugation, was redissolved in 10 ml. of water containing a small amount of NH_3 and the solution was cleared by centrifugation. This solution was shaken with 0·25 vol. of $CHCl_3$ and 0·1 vol. of amyl alcohol for 10 min. and the protein-containing chloroform-water gel removed (Sevag, Lackman & Smolens, 1938). This last procedure was repeated four times and was followed by dialysis against distilled water when some coloured material precipitated and was spun off. The nucleic acid was finally precipitated by acid ethanol, washed with 90 % ethanol, 100 % ethanol, ether, and then dried at 110°. The biuret reaction was negative.

After hydrolysis with 6 N-HCl at 120° for 6 hr. a fraction of the hydrolysed nucleic acid was chromatographed in *n*-butanol-acetic acid to detect amino-acids. The only amino-acid present other than in traces was glycine, which is, of course, derived from purines. The sugar was identified as ribose by chromatography in *n*-butanol-water-NH_3 after hydrolysis of the nucleic acid in N-H_2SO_4.

Cytidylic acid, cytidine and uridine were obtained from Dr J. Davoll. Uridylic acid was a gift from Mrs L. D. Parsons and was precipitated twice as Pb uridylate and then as ammonium uridylate. Adenine and guanine were commercial preparations.

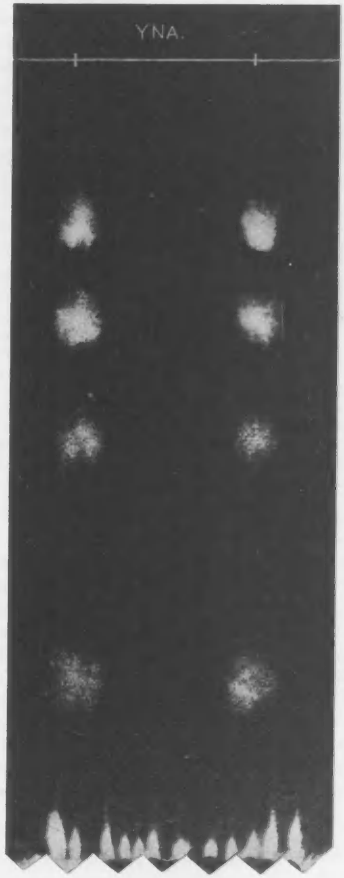

Fig. 1. Chromatogram of a hydrolysate of yeast nucleic acid. Yeast nucleic acid (YNA) was hydrolysed for 1 hr. at 100° in N-HCl. The hydrolysate was run on a chromatogram in 70 % (v/v) *tert.*-butanol containing 0·8 N-HCl and water. The spots in descending order are: (*a*) guanine, (*b*) adenine, (*c*) cytidylic acid, (*d*) uridylic acid.

RESULTS

The specimen of yeast nucleic acid used was apparently free from all but traces of protein, and contained 8·4 % phosphorus and 15·3 % nitrogen. The

optical density of a 1 cm. thickness of a solution of this nucleic acid at a concentration of 49·2 μg./ml. at pH 7·3 was 1·50 at 260 mμ. and 0·63 at 231 mμ. (at the maximum and the trough respectively). Solutions of this material, hydrolysed at 100° for 1 hr. in N-HCl and then chromatographed as described, gave rise to four major spots containing almost all the light-absorbing material which had been put on the filter paper. These spots (Fig. 1) are due to guanine, adenine, cytidylic acid and uridylic acid. The quantities of the purine bases and the pyrimidine nucleotides were estimated spectrophotometrically in the extracts of the spots. The chromatographing and estimation of this sample of nucleic acid were done in quadruplicate and the results, together with other relevant analytical data are given in Table 1.

Table 1. *Composition of yeast nucleic acid by weight*

Constituent	μg./mg. dry wt.	μ.mol./mg.
Adenine	101	0·746
Guanine	137	0·906
Cytosine	63·7	0·574
Uracil	75·4	0·674
Purines	238	1·65
'Purine-bound' ribose	239	1·59
Total bases	377	2·90
Phosphorus	84	2·71
Nitrogen	153	10·9
Nitrogen calculated from bases	151	10·8
Nucleic acid calculated from bases on basis of simple polynucleotide structure	942	—

The four spots account for 98·3 % of the nitrogen present in the nucleic acid, the remainder presumably being accounted for by nitrogenous impurities in the nucleic acid and slight loss of pyrimidine nucleotide during the hydrolysis. On a dry-weight basis 94·2 % of the material is accounted for as free nucleic acid, some of the residual 5·8 % probably being due to base combined with the acid. The standard errors of the molar ratios of the four bases were computed in the usual way, assuming that the errors are distributed randomly and these are presented in Table 2 with the corresponding molar ratios. The errors obtained are of similar magnitude to those found in the analyses of other ribonucleic acids by this method. The sources of error due to various causes may be assessed by inspection of the amounts of the substances recovered from individual spots put on the papers, and these suggest that the errors are not due entirely to pipetting.

Table 2. *Composition of yeast nucleic acid. Ratios of the four bases with standard errors*

Base	Molar ratio (mean = 1)
Adenine	1·03 ± 0·01
Guanine	1·25 ± 0·01
Cytosine	0·80 ± 0·02
Uracil	0·93 ± 0·04

DISCUSSION

While previously published methods tested by us have failed to give consistent results in the analysis of ribonucleic acids, this method has been found reliable and simple. The method of hydrolysis used is extremely mild and consequently the purines are not destroyed, while the pyrimidine nucleotides liberated are not hydrolysed further to an appreciable extent. The nucleic acid specimen used was apparently remarkably pure, its analysis comparing favourably with those of other purified yeast nucleic acids. The fact that over 98 % of the nitrogen, and virtually all of the light-absorbing material could be accounted for by the sum of the pyrimidine nucleotides and the purine bases indicates that the hydrolysis is quantitative. It will be noted in this regard that the sum of the bases, made up as it is from four estimates all having measurable standard errors, is itself subject to a standard error of about ± 1·2 %. This point is frequently disregarded in making up balance sheets of the composition of complex biological substances.

The value for free ribose, as estimated colorimetrically (Table 3), agrees within reasonable limits of error with the quantity which one would expect to find associated with the purines. The figure for phosphorus, on the other hand, is slightly lower than one would expect on the basis of a simple polynucleotide structure requiring one atom of phosphorus per molecule of base. We are inclined to attribute this to small systematic errors in the analyses rather than to postulate a branched structure for yeast nucleic acid, though the latter has been suggested by Gulland (1947). While no further evidence as to the nature of the internucleotide linkage has been obtained, it is certainly remarkably labile, and any attempt to isolate dinucleotides or small polynucleotides will necessitate novel methods of hydrolysis and estimation.

Table 3. *Composition of yeast nucleic acid. Molar ratios of constituents*

(The theoretical values are for a simple polynucleotide.)

	Found	Theory
Purine/pentose	1·04	1·00
Bases/phosphorus	1·07	1·00
Base N/total N	0·98	1·00
Purines/pyrimidines	1·32	—

In common with other nucleic acids which we have investigated, this sample of yeast nucleic acid has failed to give equimolar proportions of the bases (Table 2). This is not surprising since the methods used in the original investigations which suggested

this ratio were very crude by modern standards. What is more surprising is that this nucleic acid approximates so closely to a tetranucleotide, the maximum discrepancy being only 20%. It is evident, however, that, if all the nucleic acid particles in the preparation have a similar composition, the minimum 'molecular weight' of a polynucleotide must be at least 100,000. The inference is that the particles, as they exist in commercial yeast nucleic acid, are not all alike.

SUMMARY

A simple chromatographic method for the quantitative analysis of the bases of ribonucleic acids is described. The amount necessary for one analysis is about 0·1 mg. One hydrolysis only is needed and all the bases are recovered from single spots after chromatography on filter paper. Analyses of a specimen of yeast nucleic acid are given.

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Chromatographic Studies on Nucleic Acids

3. THE NUCLEIC ACIDS OF FIVE STRAINS OF TOBACCO MOSAIC VIRUS

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(*Received* 10 *November* 1949)

The tobacco mosaic virus was the first of several plant viruses to be shown to be nucleoprotein by the work of Bawden, Pirie, Bernal & Fankuchen (1936) and that of Bawden & Pirie (1937). The importance of the nucleic acid, although originally disputed by workers in America, is now accepted as a matter of course, and it seems probable that nucleic acid is an essential factor for the multiplication of many viruses. In their original work Bawden & Pirie (1937) isolated the nucleic acid in several ways and showed that it resembled the ribose nucleic acid of yeast. Later Loring (1939) showed that its constituents were at least similar to those in yeast nucleic acid. Recently we have developed an accurate quantitative method for the analysis of ribonucleic acids (Smith & Markham, 1950), and it seemed desirable to see whether the virus nucleic acids resembled yeast nucleic acid in their quantitative composition, and to what extent, if at all, the various viruses differ in the make-up of their nucleic acids. In this paper we discuss the results obtained with five different strains of virus, all of which have serological groupings in common and all of which are similar morphologically.

EXPERIMENTAL

Materials

The viruses used in this investigation were all related strains belonging to the tobacco mosaic virus group. All were passed through single lesions on *Nicotiana glutinosa* (excepting cucumber virus 4 which does not go to this plant) in order to avoid any possibility of strain mixtures. The purification method used followed the lines of that of Bawden & Pirie (1937).

The strains used were as follows:

Tobacco mosaic. The ordinary type strain, which has been maintained here for many years, has from time to time been put through single lesions to maintain its purity. It does

produce a small percentage of yellow and necrotic yellow mutants. It was multiplied on White Burley tobacco plants.

Tomato mosaic 844 A. This virus, obtained from Dr R. E. Taylor of the National Agricultural Advisory Service, is the commonest mosaic disease of glasshouse tomatoes in this country. It resembles in its symptoms on tomato the type virus, but differs in that it does not cause a systematic disease in tobacco. It was grown on Potentate tomatoes.

Tomato aucuba mosaic. The original 'Aucuba' mosaic of tomato (Henderson Smith, 1928) appears to differ from that so named by American workers. This virus is a frequent cause of disease in commercial tomato plants in this country. It gives a very characteristic symptom pattern in tomatoes and causes only local necrotic lesions in tobacco plants. It was obtained through Dr R. E. Taylor and was grown on Potentate tomato plants.

Rib-grass virus. This strain of tobacco mosaic was found on plantain by Holmes (1941). It was obtained through Dr G. Oster, and was grown on Kawala tobacco plants.

Cucumber virus 4. This virus, which is apparently confined to cucurbitaceous plants, was first described in this country, where it now no longer seems to occur naturally (Ainsworth, 1935). It was obtained through Dr R. E. F. Matthews and grown on Bedfordshire Ridge cucumber plants.

Preparation of the virus nucleic acids. The nucleic acids from all the virus strains were isolated in an identical manner. A solution of the virus at pH 7–8 containing a trace of salt was heated at 100° for about 10 min. when the virus protein is denatured and collects as a flocculent precipitate, while the nucleic acid remains in solution. As the solution often became slightly acid on boiling, the pH was readjusted to 7–8 before the protein was centrifuged off. The nucleic acid was precipitated from the supernatant fluid by the addition of an equal volume of ethanol and a few drops of N-HCl. This precipitate was collected by centrifugation and dissolved in the minimal quantity of very weak NH_3 and centrifuged at 12,000 rev./min. to remove traces of protein. The nucleic acid was finally precipitated a second time with acidic ethanol in a small tube, and collected by centrifugation as a pellet which could then be hydrolysed directly in the centrifuge tube.

Methods

Nucleic acid analysis. The technique employed has been described previously (Markham & Smith, 1949; Smith & Markham, 1950). The virus nucleic acid was hydrolysed for 1 hr. at 100° in sufficient N-HCl to give a concentration of about 20 mg. of nucleic acid/ml. We have shown that this hydrolysis is quantitative, and as the bases are removed quantitatively from the chromatograms, it is unnecessary to use large quantities of nucleic acid in cases like this where only knowledge of the relative proportions of the bases is sought. The hydrolysate containing the purine bases and the pyrimidine nucleotides was placed in measured quantities (10–20 μl.) on the chromatogram paper. This was then run in 70% (v/v) *tert.*-butanol-water, 0·8 N with respect to HCl, the absorbing areas were located by our ultraviolet photographic technique, cut out together with adjacent blank areas, eluted in 5 ml. of 0·1 N-HCl and estimated on the Beckman photoelectric spectrophotometer making use of the characteristic ultraviolet absorption. The same spectrophotometric data were used as in our analysis of yeast nucleic acid (Smith & Markham, 1950).

Chromatography of the sugar component. The purine-bound pentose was liberated from the nucleic acid by hydrolysis at 100° in N-H_2SO_4. Sulphate was removed from the hydrolysate by neutralization with $Ba(OH)_2$ followed by centrifugation. The solution, which contained pentose, was concentrated by vacuum distillation and placed on a paper chromatogram together with suitable markers (arabinose, rhamnose and D-ribose from yeast nucleic acid). This chromatogram was run in *n*-butanol-water-NH_3, a solvent which clearly resolves ribose from other known pentoses (Partridge, 1948). The sugars were located on the paper by spraying with aniline oxalate and heating for 10 min. at 100° (Partridge, 1949).

RESULTS

Analysis of nucleic acids

Ultraviolet absorption of the nucleic acids. The absorption curves of the nucleic acids in the ultraviolet region were plotted from measurements made using the Beckman photoelectric spectrophotometer. These curves were obtained as a routine check on the strength of the nucleic acid preparations and were used as a guide to the quantity necessary for a hydrolysis. As shown in Fig. 1, these curves have a

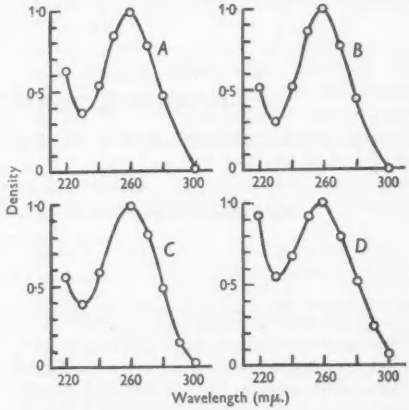

Fig. 1. Ultraviolet absorption curves of the nucleic acids from four strains of tobacco mosaic virus. Density plotted against wavelength in mμ. The concentration is approximately 35 μg. of nucleic acid/ml. in a 1 cm. cell. Strains: *A*, tobacco mosaic; *B*, rib-grass; *C*, tomato mosaic; *D*, cucumber 4.

similar although not identical shape. All show a well defined peak at 260 mμ. and a trough at about 230 mμ. It has been suggested that the ratio peak density/trough density can be used as a criterion of purity of nucleic acids, but we consider that this ratio is also largely dependent on the degree of aggregation of the particles which affects their light scattering in this region.

Identification of the components of the nucleic acids. On hydrolysis with N-HCl at 100° for 1 hr. the nucleic acids from all the tobacco mosaic virus strains yield only adenine, guanine, cytidylic and uridylic acids, which were identified by their relative positions on the chromatograms, by absorption spectra and by hydrolysis of the nucleotides to the free bases. Like all other ribonucleic acids so far investigated these, therefore, contain only the purines adenine and guanine and the pyrimidines cytosine and uracil. The purine-bound pentose from the type strain and that from tomato mosaic were identified by paper chromatography and found to be identical with ribose from yeast nucleic acid.

Quantitative composition. Using our technique we have determined the molar ratios of the four purine and pyrimidine bases in each of the nucleic acids. These ratios are given in Table 1. As the tomato mosaic virus nucleic acid most nearly approached the mean values for all five strains, comparisons are made with the values obtained from this nucleic acid. Those figures which appear to differ significantly from the tomato mosaic values are printed in heavy type. (The significances of the values were computed by means of the *t* test. In three cases the value of *P* was less than 0·01 and in the fourth (the uracil of cucumber 4) *P* was between 0·01 and 0·02.)

Table 1. *Purine and pyrimidine composition of the nucleic acids from five strains of tobacco mosaic virus*

(The molar proportions of the four bases (mean = 1) together with their S.E.M. are given. The figures in heavy type differ significantly from the corresponding values for tomato mosaic virus nucleic acid.)

Strain	Adenine	Guanine	Cytosine	Uracil	No. of determinations
Aucuba mosaic	1·20	0·95	0·78	1·05	5
Type tobacco mosaic	1·24±0·026	**1·17**±0·023	**0·62**±0·021	0·96±0·019	4
Rib-grass	1·17	1·08	0·69	1·05	6
Cucumber 4	**1·04**±0·009	1·03±0·009	0·74±0·006	**1·19**±0·012	4
Tomato mosaic	1·18±0·007	1·04±0·014	0·73±0·004	1·05±0·037	4
Mean of all strains	1·17	1·05	0·71	1·06	23
Yeast nucleic acid	1·03	1·25	0·80	0·93	11

Serological and biological tests

As the tomato virus differed from the tobacco mosaic virus in the composition of its nucleic acid we made some serological cross-absorption experiments. The antisera used were produced by injecting into the ear veins of each rabbit a single quantity of 1–2 mg. of purified virus in a volume of 1 ml. The rabbits were bled after a fortnight, and the sera were titrated against the heterologous strain by the optimal proportions method (i.e. antitobacco mosaic against tomato mosaic virus and antitomato mosaic against tobacco mosaic virus). A quantity of each serum was then incubated at 50° with an excess of the heterologous virus strain and the precipitate centrifuged off. The absorbed sera were then tested against more of the absorbing virus for precipitins to check the completeness of the absorption and finally against the homologous virus to detect the presence of antigenic components not shared by the pair of viruses. Absorbed sera were also tested against cucumber virus 4, because the latter is probably the most atypical virus of this group. It will be seen from the results shown in Table 2 that the tobacco mosaic and cucumber 4 viruses have antigenic components which the tomato mosaic virus lacks. Tomato mosaic also has an antigenic component which is not present in tobacco mosaic, but which is shared by cucumber virus 4.

Infection with one strain of a virus protects a plant from subsequent infection with a related strain (Salaman, 1933). This phenomenon of 'cross-protection' has been made the basis of a method which is in general use among plant virus workers for determining whether two viruses are strains of the same virus or are not related to each other; e.g. a plant infected with a virus suspected of being a strain of cucumber mosaic virus might be inoculated with a strain of the latter which has easily distinguishable symptoms. Failure of the authentic strain to enter the plant is regarded as proof of a strain relationship between the two. Recently it has been found that this cross-protection fails in some cases where serological and other tests have definitely established a relationship, and in these cases it seems likely that the relationship is fairly remote. A similar case exists here since the difference in antigenic constitution between tomato mosaic and tobacco mosaic is paralleled by the fact that the

Table 2. *Serological cross absorptions*

(+ indicates precipitation; − indicates absence of precipitation.)

Serum	Tobacco mosaic	Tomato mosaic	Cucumber 4
Antitobacco mosaic	+	+	+
Antitobacco mosaic absorbed with tomato mosaic	+	−	+
Antitomato mosaic	+	+	+
Antitomato mosaic absorbed with tobacco mosaic	−	+	+

former is able to infect tomato plants already systematically infected with tobacco mosaic virus (Hitchborn, private information).

On the other hand, Holmes (1941) found that there was a cross-protection relationship between his rib-grass virus and tobacco mosaic virus. These facts suggest that the English tomato mosaic viruses may be even more distantly related to tobacco mosaic virus than is the rib-grass virus, which because of its differences in host range and symptomatology is generally regarded as a rather exceptional strain.

DISCUSSION

The nucleic acid of the tobacco mosaic virus has been investigated by Loring (1939) and Schwerdt & Loring (1947), who from acid hydrolysates obtained and identified adenine, guanine and cytosine, and after alkaline hydrolysis isolated guanylic, cytidylic and uridylic acids which were identified by their chemical composition, optical rotations and decomposition points. Although no direct evidence was offered to show that the sugar was actually ribose, their results indicated that the virus nucleic acid was similar to that of yeast from a qualitative point of view.

As nucleic acid of some kind has been found as an invariable constituent of all authentic virus preparations, and is probably responsible in part for the biological activity of these parasites, it is of some interest to determine whether any specific differences can be found in the nucleic acids of viruses which could be correlated with the biological differences of their nucleoproteins. In the case of the protein component of various strains of tobacco mosaic virus it was found by Knight and others (Knight, 1942, 1943, 1947, 1949; Knight & Stanley, 1941; Gaw & Stanley, 1947) that appreciable differences in the amino-acid composition can be detected between the more distantly related strains. In the case of the nucleic acids there are fewer factors that can vary, and as the components which we have found seem to be the same as those of yeast nucleic acid, there is only the possibility of measuring the variation in the relative amounts of the four nucleotides present. Possibly at a later date information about the arrangement of the nucleotides relative to each other may be obtained. It is striking that the relative proportion of the four nucleotides is so similar in all five strains, only four small differences having been noticed. It is possible that more refined measurements on a very large number of preparations may show up more differences of an even smaller magnitude. The general composition of the nucleic acids of the tobacco mosaic group, however, is considerably different both from that of a yeast nucleic acid, which we have investigated (Smith & Markham, 1950) and from that of several preparations of the nucleic acid of the turnip yellow mosaic virus (Kenneth Smith, Markham & Smith, unpublished). It may be found that in general closely related viruses have very similar nucleic acids and we intend to investigate this point further.

We should point out here that there is little probability that, apart from the rib-grass strain and cucumber 4, our viruses are the same as those with similar names on which amino-acid determinations have been made. Indeed our type strain of virus has a glutamic acid content (Wiltshire, unpublished) which is entirely different from that of the type strain investigated by Knight. This state of affairs is inevitable as there would appear to be an almost infinite number of strains of this virus, most of which are in a state of flux, and there is little one can do to prevent changes due to mutations becoming dominant in the host plants. It is for this reason that we have been careful to ensure that our experimental material originated from single necrotic lesions and was therefore relatively homogeneous.

SUMMARY

1. The nucleic acids from five distinct strains of tobacco mosaic virus have been isolated and their composition studied by means of paper chromatography.

2. The purine-bound sugar from the nucleic acids of two of these strains has been shown by paper chromatography to be identical with ribose from yeast nucleic acid.

3. All the nucleic acids have been shown by our chromatographic technique to contain only the four bases, adenine, guanine, cytosine and uracil.

4. The nucleotide compositions of the nucleic acids from the five strains were found to be very similar. Small significant differences were detected among three of the strains.

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Effect of Acetate on Glycogen Synthesis and Glucose Utilization in the Isolated Diaphragm of Rats

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Central metabolic products are believed to be basic regulators of cellular metabolism, the influence of the hormones on metabolism being only secondary, or superimposed on these primary regulatory mechanism. It may be of interest therefore to investigate the influence of such products on certain metabolic processes *in vivo* and *in vitro*. Bloch (1947) has pointed out the central position of acetic acid in metabolism. In this paper we made a study of the influence of acetate on glycogen synthesis and glycogenolysis and glucose utilization by isolated rat diaphragms, using the technique of Gemmill (1940*a*, *b*).

EXPERIMENTAL

Male albino rats of laboratory stock weighing 80–100 g. were used. Preparation of diaphragm was carried out according to Gemmill (1940*a*), using diaphragms from rats fasted for 20 hr. The medium used for incubation was 'phosphate-buffered saline' containing 0·74, NaCl; 0·015, Na_2HPO_4 and 0·003% NaH_2PO_4. All the added substrates were given as Na salts. The vessels containing the diaphragms in the medium were gassed for 2 min. with O_2 and then placed in a water bath at 37°. The flasks were shaken 120 times/min.

Glycogen synthesis. This was measured according to Tuerkischer & Wertheimer (1948).

Glucose utilization. For measurement of the glucose utilization by the diaphragm the methods of Krahl & Cori (1947) and Perlmutter & Greep (1948) were used.

Measurements of the glycogen synthesis by the isolated diaphragm of acetate-fed rats. The rats were given the following diet during 2 days: 58% carbohydrate, 12% Na acetate, 20% casein and 10% olive oil, with the usual addition of vitamins and salts. After fasting for 24 hr. the animals were killed, the diaphragms excised and the experiments carried out in the usual medium with 0·05% glucose.

Measurement of respiration. Warburg's direct method was used for the determination of the O_2 uptake (Dixon, 1943) in phosphate Ringer solution. The diaphragms were halved, one half was incubated in the medium containing 0·05% glucose and 0·1% acetate and the other half with glucose alone. Measurements were made over periods of 3 hr. and the average value for 1 hr. was recorded.

Chemical procedures. Determinations of glycogen in the diaphragm were carried out by a micromodification of Pflüger's method (Good, Kramer & Somogyi, 1933). Glucose was estimated according to Somogyi (1937). Determination of acetic acid was carried out by steam distillation.

RESULTS

Glycogen synthesis by isolated rat diaphragm, with acetate in the medium

Incubation of rat diaphragm in the usual medium was followed by a marked glycogenolysis (Tuerkischer & Wertheimer, 1948). On addition of lactate (0·1%), citrate (0·1%) and succinate (0·1%) there was glycogenolysis of the same order of magnitude as in the medium without additional substrate. α-Ketoglutarate decreased, while acetoacetate markedly increased the glycogenolysis. Acetate in a concentration of 0·2% caused glycogenolysis of the same order of magnitude as was observed in the medium without substrate ($-0{\cdot}062 \pm 0{\cdot}009$ in three experiments), but no glycogenolysis was observed in the presence of 0·05% acetate ($-0{\cdot}002 \pm 0{\cdot}010$ in four experiments). Addition of 0·1% acetate gave rise to a small but significant net glycogen increase. Pyruvate caused net increase in glycogen, though not to the same extent as glucose (Table 1).

Table 1. *Glycogen synthesis by normal rat diaphragm in presence of acetate, pyruvate, lactate, etc.*

(The diaphragms were incubated at 37° for 3 hr. Glycogen synthesis is expressed as mg. glucose/100 mg. wet tissue.)

No. of experiments	Medium containing (0·1%)	Glycogen synthesis (mg./100 mg.)
14	Acetate	$0{\cdot}016 \pm 0{\cdot}008$
11	Pyruvate	$0{\cdot}037 \pm 0{\cdot}013$
7	Lactate	$-0{\cdot}062 \pm 0{\cdot}023$
5	Citrate	$-0{\cdot}060 \pm 0{\cdot}026$
5	Succinate	$-0{\cdot}069 \pm 0{\cdot}024$
7	α-Ketoglutarate	$-0{\cdot}020 \pm 0{\cdot}019$
4	Acetoacetate	$-0{\cdot}115 \pm 0{\cdot}024$

Influence of acetate on glycogen synthesis from glucose

Glycogen synthesis by rat diaphragm from 0·05 % glucose was markedly increased by addition of 0·1 % acetate. This increase exceeded by far the sum of the values of synthesis caused by each of the substrates separately. This effect of acetate depended on the concentration of glucose in the medium. In presence of 0·1 % glucose, this increase was smaller but still significant as compared with 0·1 % glucose alone, while with 0·2 % glucose the values of glycogen synthesis were the same with and without acetate. With glucose concentration below 0·05 % the effect of acetate could again be demonstrated (Table 2). not increase glycogen synthesis from glucose. With 0·2 % acetate and 0·05 % glucose the values found were $0·100 \pm 0·029$ (four experiments), while for 0·05 % acetate and 0·05 % glucose a value of $0·047 \pm 0·039$ (four experiments) was found. A 3 hr. incubation was chosen, since 45 min. values were far below the optimum, while after 5 hr. the amount of glycogen synthesis decreased. Lactate, succinate, citrate, propionate and acetoacetate in concentrations of 0·1 % added to a medium containing 0·05 % glucose did not increase the synthesis of glycogen; some of these substances—citrate and especially acetoacetate—inhibited glycogen synthesis from glucose (Fig. 1). A marked increase of glycogen synthesis from glucose could be demon-

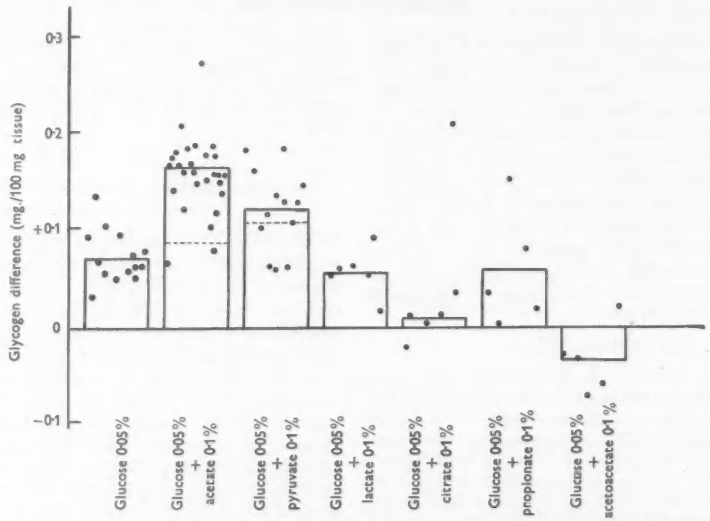
Glycogen difference (mg./100 mg. tissue)
0·3
0·2
+0·1
0
−0·1
Glucose 0·05 %
Glucose 0·05 % + acetate 0·1 %
Glucose 0·05 % + pyruvate 0·1 %
Glucose 0·05 % + lactate 0·1 %
Glucose 0·05 % + citrate 0·1 %
Glucose 0·05 % + propionate 0·1 %
Glucose 0·05 % + acetoacetate 0·1 %

Fig. 1. Effect of various substances on glycogen synthesis of rat diaphragm. – – –, theoretical sum of the values for glucose and other substances acting separately.

Table 2. *Effect of acetate on glycogen synthesis from varying glucose concentrations*

(The diaphragms were incubated at 37° for 3 hr. Glycogen synthesis is expressed as mg. glucose/100 mg. wet tissue.)

No. of experiments	Medium containing: Glucose (%)	Medium containing: Acetate (%)	Glycogen synthesis (mg./100 mg.)
14	0·05	—	$0·072 \pm 0·007$
28	0·05	0·1	$0·166 \pm 0·010$
14	0·1	—	$0·109 \pm 0·020$
6	0·1	0·1	$0·161 \pm 0·014$
6	0·2	—	$0·166 \pm 0·027$
6	0·2	1·0	$0·163 \pm 0·024$

The acetate concentration of 0·1 % seemed to be optimal, as both higher and lower concentrations did strated on addition of 0·1 % α-ketoglutarate only with diaphragms of fed rats. There was no increase in diaphragms from rats fasted for 24 hr. With diaphragms from fed rats the glycogen synthesis was $0·155 \pm 0·026$ (seven experiments); diaphragms from fasted rats gave values of $0·073 \pm 0·011$ (twelve experiments). Addition of pyruvate caused only a slight increase of glycogen synthesis, the value being not significantly higher than the sum of the values for pyruvate and glucose acting separately.

There was a further increase in the synthesis of glycogen from glucose in presence of acetate when insulin was added to the medium. The synthesis in the presence of insulin was $0·221 \pm 0·024$ (eleven experiments) as compared with $0·166 \pm 0·010$ (twenty-eight experiments) without insulin. The effect of

acetate on glycogen synthesis also could be demonstrated when serum was used instead of phosphate-buffered saline. When diaphragms were incubated in serum the difference value (Tuerkischer & Wertheimer, 1948) with and without acetate was $0{\cdot}048 \pm 0{\cdot}015$ (four experiments).

Influence of acetate on glucose utilization by rat diaphragm

The glucose uptake by rat diaphragm was markedly decreased in presence of 0·1 % acetate. There was also a decrease in the oxidation of glucose as measured in the Warburg apparatus (Table 3). The

Table 3. *The effect of acetate on the oxygen uptake of the rat diaphragm*

(One half of the diaphragm was incubated in phosphate Ringer containing 0·05 % glucose, the other half in the same medium with addition of 0·1 % acetate.)

Exp. no.	Composition of medium	O_2 uptake (µl.)
1	Without acetate	−3·85
	With acetate	−3·10
2	Without acetate	−4·05
	With acetate	−3·26
3	Without acetate	−4·11
	With acetate	−3·32

quotient, glycogen synthesis/glucose utilization rose from 0·12 for 0·05 % glucose alone, to 0·47 for 0·05 % glucose and 0·1 % acetate. This effect of acetate was also observed when acetate was previously given *in vivo*. Rats were fed an acetate-containing diet, fasted for 24 hr. and their diaphragms incubated with 0·05 % glucose without further addition of acetate *in vitro*. In these experiments a quotient of 0·32 was found.

This decrease in the glucose uptake could not be demonstrated in a medium with higher concentrations of glucose. The quotient, glycogen synthesis/glucose utilization, with 0·2 % glucose alone was 0·25; when acetate was added this value remained unchanged. In a medium containing 0·05–0·1 % glucose, the disappearance of acetate was $0{\cdot}310 \pm 0{\cdot}042$ mg./100 mg. of diaphragm. No definite relation was found between the amount of acetate which disappeared from the medium and the amount of glucose utilized during the incubation of the diaphragm (Table 4).

DISCUSSION

Influence of acetate on glycogen synthesis. The absolute value for net glycogen increase from acetate by the muscle is small but significant. Considering that other substances such as lactate, citrate, succinate, ketoglutarate, propionate and acetoacetate do not show this effect, but on the contrary even cause marked net decrease of glycogen, the significance of the findings with acetate become enhanced. These experiments do not establish whether the acetate itself is incorporated into the glycogen molecule, or whether its participation in glycogen synthesis is indirect. This can be established only by experiments with labelled acetate. It should be stressed, however, that besides glucose only acetate and pyruvate gave rise to net glycogen increase, while all other substrates caused net glycogen decrease.

Gemmill (1940*a*, *b*) had found that the diaphragm cannot use lactic acid for synthesizing glycogen. This finding is in accord with the generally accepted belief that in mammals most of the lactic acid is transported by way of the blood to the liver, and only there resynthesized to glycogen (Cori cycle). Our findings demonstrate that muscle glycogen can be synthesized in the presence of some shorter carbon chains, as well as from glucose.

Influence of acetate on glycogen synthesis from glucose and on glucose utilization. Our main task was to investigate the influence of acetate on carbohydrate metabolism of the isolated diaphragm. Glucose utilization on the whole decreases, the glucose oxidation as measured in the Warburg apparatus is lowered, while glycogen synthesis increases. The quotient, glycogen synthesis/glucose utilization, is therefore markedly increased. Thus acetate influences carbohydrate metabolism by enhancing synthesis on the one hand, and lowering oxidation on the other. It should be noted, however, that this acetate effect can be demonstrated only when glucose concentrations are below the normal

Table 4. *Effect of* 0·1 % *acetate on glycogen synthesis and glucose utilization by rat diaphragm*

(The diaphragms were incubated at 37° for 3 hr. Glycogen synthesis and glucose utilization are expressed as mg. glucose/100 mg. wet tissue.)

No. of experiments	Medium containing: Glucose (%)	Medium containing: Acetate (%)	Glycogen synthesis (mg./100 mg.)	Glucose utilization (mg./100 mg.)	Glycogen synthesis / Glucose utilization
10	0·05	—	$0{\cdot}066 \pm 0{\cdot}006$	$0{\cdot}558 \pm 0{\cdot}031$	0·12
14	0·05	0·1	$0{\cdot}147 \pm 0{\cdot}008$	$0{\cdot}342 \pm 0{\cdot}013$	0·47
12	0·05	*In vivo**	$0{\cdot}126 \pm 0{\cdot}016$	$0{\cdot}463 \pm 0{\cdot}033$	0·32

* Rats were fed for 2 days a diet containing 12 % acetate and fasted afterwards for 24 hr.; no acetate was added *in vitro*.

physiological concentration. With glucose concentration above 0·1 %, neither enhanced synthesis nor lowered utilization was observed. When looking for an explanation of this effect, it should be noted that when the acetate is given *in vivo*, and the diaphragm isolated 24 hr. after the last acetate feeding, the effect on glycogen synthesis and glucose utilization is the same as when the acetate is given *in vitro*. Acetate and glucose might be considered competitors in the tricarboxylic acid cycle. But since the level of glycogen synthesis *in vitro* remains high and that of general glucose utilization low, even 24 hr. after the acetate feeding was discontinued, this explanation is not acceptable.

According to these experiments, acetate in presence of low glucose concentrations causes changes of long duration in the muscle cell, presumably by sparing carbohydrate and increased metabolism of fat. The exact mechanism of this action is not known, but this appears to be the only explanation for the fact that the action of the acetate given *in vivo* can still be demonstrated 24 hr. later in the isolated diaphragm. It seems, therefore, that acetate can influence cell metabolism in a definite direction. No similar effect on the carbohydrate metabolism of the diaphragm was shown by any of the other substrates examined. Even pyruvate, which itself brings about net glycogen increase in the diaphragm, did not increase significantly glycogen synthesis from glucose.

Insulin markedly enhances glycogen synthesis in media containing acetate and glucose; this increase, however, can be explained by the enhancing action of insulin on the glycogen synthesis from glucose alone.

SUMMARY

1. Rat diaphragm in phosphate-buffered saline shows a significant net glycogen increase when incubated with 0·1 % acetate or pyruvate. When diaphragm is incubated in the medium without any addition, glycogenolysis takes place; this glycogenolysis is not affected by addition of lactate, citrate or succinate. Acetoacetate increases, whilst ketoglutarate decreases glycogenolysis.

2. Acetate (0·1 %) and 0·05 % or less glucose increase glycogen synthesis to a much greater extent than would be expected from the sum of the synthesis values for each substrate alone. Total glucose utilization and glucose oxidation are markedly lowered. The ratio of glycogen synthesis/glucose utilization therefore increases. No other substrate mentioned above has a similar effect. With 0·2 % glucose the synthesis of glycogen and the utilization of glucose are not affected by addition of acetate.

3. Acetate given *in vivo* causes, after an interval of 24 hr., an increase in glycogen synthesis from glucose and a decrease in glucose utilization of the isolated rat diaphragm *in vitro*.

4. Acetate is assumed to effect primary cellular regulation of metabolism.

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Effect of Acetate on the Glycogen Synthesis *in vivo*

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In experiments of the preceding paper it was shown that acetate at certain concentrations influences carbohydrate metabolism of the isolated rat diaphragm, providing that the glucose concentration does not exceed 0·1 %. The *in vitro* acetate effect, which consists of increased glycogen synthesis and decreased glucose utilization, was present even in diaphragms of rats which had previously been fed a diet containing acetate and then killed after a 24 hr. fast. When these diaphragms were incubated with 0·05 % glucose, without further addition of acetate, there was increased glycogen synthesis and decreased glucose uptake.

In the following experiments the influence of acetate on carbohydrate metabolism *in vivo* was investigated. Our starting point was the following

experiment. Rats fed a diet containing 12% acetate show after a 24 hr. fast more glycogen in the liver and muscle than controls fed a diet containing an equivalent amount of carbohydrate.

EXPERIMENTAL

Animals and diet. Male albino rats of our stock, weighing 80–120 g., were used. For at least 2 days before the experiment they received the following diet: 70% carbohydrate, 20% casein, and 10% fat, with the usual addition of salts and vitamins. After this the experimental animals were given for at least 2 days a diet consisting of 58% carbohydrate, 12% acetate, 20% casein, and 10% fat, and the usual additions. The controls continued to get the 70% carbohydrate diet. In some of the experiments paired feeding was used. This was, however, discontinued when it was found that acetate had no influence on the amount of food eaten.

The protein-rich diet contained 58% casein, 12% acetate, 20% carbohydrate and 10% fat, salts and vitamins, while the corresponding control diet contained 70% casein, 20% carbohydrate and 10% fat.

In the phlorrhizin experiments the rats were fasted for 24 hr. and 30 mg. phlorrhizin were injected subcutaneously during the fasting period. In a number of the experiments the animals were killed 3 hr. after the phlorrhizin administration, while in other experiments the rats were killed 17 hr. after the administration of phlorrhizin, i.e. following a recovery period. The chemical procedures used were the same as described in the previous paper. Alloxan diabetes was induced by intravenous injection of 6–8 mg. of alloxan/100 g. rat.

RESULTS

After fasting for 24 hr., acetate-fed rats showed more glycogen in liver and muscle than controls. If the acetate diet was given for 1 day instead of the usual 2 days the differences were less marked. Acetate (6 or 3%) gave nearly the same results as 12% acetate. With a 1% acetate concentration the effect was abolished (Table 1).

The influence of the duration of the fasting period. The glycogen contents of liver and muscle were similar in both acetate-fed and control rats, if the animals were not previously fasted. After an 8 hr. fast the glycogen content decreased in both, but no differences were observed between the acetate-fed rats and the controls. After 18 hr. fasting there was even more glycogen in the livers of the control, than in those of the acetate-fed animals. Fasting periods of 32 and 48 hr. gave the same results as the initial experiment. With a 72 hr. fast again no significant differences could be observed between the acetate-fed and the control rats.

Other substances. Addition of 6–12% propionate, butyrate or stearate to the diet had no influence on the liver and muscle glycogen. With similar amounts of lactate or citrate, the glycogen content, after a 24 hr. fast, was even lower in the experimental rats than in the controls (Table 2).

The glycogen content, 3 hr. after the administration of phlorrhizin, reaches very low values in the

Table 1. *Glycogen after a 24 hr. fast, as influenced by the acetate concentration in the diet*

No. of experiments	Acetate added to diet (%)	Glycogen of liver (as mg. glucose/g. wet tissue)		Glycogen of muscle (as mg. glucose/g. wet tissue)	
		With acetate	Without	With acetate	Without
12	12	0·41±0·081	0·12±0·030	0·27±0·018	0·21±0·018
7	6	0·42±0·079	0·11±0·013	0·22±0·014	0·19±0·011
6	3	0·44±0·16	0·13±0·028	0·26±0·042	0·19±0·011
5	1	0·16±0·028	0·17±0·048	0·18±0·039	0·18±0·011

Table 2. *Liver and muscle glycogen, following a 24 hr. fast after a diet containing lactate, citrate, propionate, butyrate or stearate*

No. of experiments	Addition to diet (%)	Glycogen (as mg. glucose/g. wet weight)			
		Liver		Muscle	
		With acetate	Without*	With acetate	Without
5	Lactate 12	0·22 ±0·067	0·49±0·020	0·28±0·053	0·28±0·040
5	Citrate 12	0·15 ±0·044	0·31±0·061	0·30±0·010	0·26±0·030
8	Propionate 6–12	0·077±0·021	0·14±0·035	0·16±0·028	0·14±0·017
6	Butyrate 6–12	0·22 ±0·075	0·18±0·032	0·16±0·017	0·17±0·017
5	Stearate 12	0·26 ±0·058	0·24±0·028	0·22±0·021	0·20±0·013
12	Acetate 12	0·41 ±0·081	0·12±0·030	0·27±0·018	0·21±0·018

* The 24 hr. fasting values of liver glycogen of normal rats usually vary greatly in different seasons. This work was carried out during summer, fall and winter.

Table 3. *The acetate effect on liver and muscle glycogen as influenced by the duration of the fast*

No. of experiments	Duration of fast (hr.)	Glycogen (as mg. glucose/g. wet weight)			
		Liver		Muscle	
		With acetate	Without	With acetate	Without
5	0	1·54±0·18	1·69±0·20	0·41±0·037	0·39±0·036
5	8	0·58±0·13	0·51±0·22	0·30±0·022	0·24±0·017
5	18	0·27±0·072	0·52±0·20	0·32±0·046	0·34±0·019
12	24	0·41±0·081	0·12±0·30	0·27±0·018	0·21±0·018
5	32	0·30±0·055	0·08±0·026	0·19±0·017	0·23±0·023
6	48	0·49±0·10	0·14±0·036	0·20±0·040	0·14±0·029
5	72	0·35±0·11	0·28±0·12	0·11±0·031	0·12±0·022
5	24 (3 hr. after phlorrhizin)	0·04±0·009	0·06±0·018	0·13±0·029	0·14±0·021
5	24 (17 hr. after phlorrhizin)	0·38±0·076	0·12±0·049	0·26±0·010	0·20±0·035

acetate-fed animals as well as in their controls. After a recovery period of 17 hr. the differences in the amount of glycogen of experimental rats and controls were about the same as in the 24 hr. fast experiment (Table 3).

The influence of acetate on the glycogen of animals fed a protein-rich diet. Acetate caused an additional increase of the already high glycogen values (the 'protein effect' described by Mirski, Rosenbaum, Stein & Wertheimer, 1938); the liver glycogen, after a 24 hr. fast, was 1·18 ± 0·12 in the acetate-fed rat as compared with 0·77 ± 0·17 in the protein-fed controls; the muscle glycogen was 0·29 ± 0·032 as compared with 0·23 ± 0·021 (five experiments).

The influence of acetate on the glycogen content of alloxan diabetic rats. The effect of acetate on liver and muscle glycogen could be demonstrated in alloxan diabetic animals (three experiments).

DISCUSSION

In the preceding *in vitro* experiments, 0·1 % acetate was found to increase the glycogen synthesis and decrease the glucose utilization by the isolated diaphragm in presence of 0·1 % or less glucose. We might assume that acetate in the presence of a normal or lowered blood glucose concentration causes a decrease in carbohydrate utilization and therefore, in spite of the fast, the glycogen content remains high, a shift in metabolism from carbohydrate to fat utilization having taken place. This could explain the *in vivo* as well as the *in vitro* experiments. However, other factors which might be responsible for the high glycogen content of the acetate-fed animals must be taken into consideration.

An alkalosis effect could be excluded as, according to McKay, Wick, Carne & Barnum (1941), alkali administration depresses the liver glycogen and enhances the ketonaemia. Furthermore, if alkalosis were responsible for the high glycogen values, citrate and lactate should have the same effect as acetate. We found, however, that citrate and lactate depressed the glycogen values.

Acetate itself could be an excellent glycogen precursor, or, being an available energy source, might have a carbohydrate-sparing action, and thus be responsible for an enhanced glycogen synthesis. If this were the case, differences in the glycogen content would be expected after short fasting period. Differences were observed, however, only after longer fasts.

Acetate might cause an inhibition of glycogenolysis. Here again the absence of differences in glycogen content after short fasting periods does not fit in with this assumption. Moreover, after phlorrhizin administration, the glycogen drops to about the same level in both the acetate-fed animals and the controls, and only after a long recovery period do the differences reappear.

Acetate may cause enhanced gluconeogenesis. This assumption is supported by the above-mentioned finding that after phlorrhizin the liver-glycogen values drop to minimal traces, in the acetate-fed rat as well as in the controls. Only after a recovery of 17 hr., i.e. time enough for neogenesis, do the differences reappear. In further support of this assumption is the fact that after short fasting periods the glycogen content in the livers of acetate-fed animals is even lower than in the livers of the controls, and only after a fasting period of 24–48 hr. do the glycogen values in the acetate-fed rats become much higher than in the controls. After a fasting period of 72 hr. the differences disappear, as by then gluconeogenesis has been initiated in the controls as well. There was, however, no consistent increase in nitrogen excretion during the fasting period in the acetate-fed animals. This point therefore still remains to be cleared up. After taking into consideration all these points, it appears that the explanation offered in the *in vitro* experiments is suitable in the case of the *in vivo* experiments as well. In the latter there may be gluconeogenesis as

well as lowered glucose utilization and enhanced glycogen synthesis. The fact that acetate administered 24 hr. beforehand, *in vivo*, causes enhanced glycogen synthesis, and a drop in the glucose utilization in the isolated diaphragm confirms the explanation offered at the beginning of the discussion.

According to Todd, Barnes & Cunningham (1947), addition of glycine to the normal diet is followed by a higher fasting liver glycogen as compared with the corresponding controls. When insulin is administered to these animals the drop in the liver and muscle glycogen and in the blood sugar is markedly smaller than in rats kept on a normal diet (Cunningham, Barnes & Todd, 1948). This phenomenon is in accordance with the so-called 'protein effect' described by Mirski *et al.* (1938). It remains to be seen whether a connexion exists between the acetate effect and the glycine action.

Acetate and cellular regulation. In this connexion it should be mentioned that when rats were fed a protein-rich diet (Mirski *et al.* 1938) or a diet containing mostly fat (Stein, Tuerkischer & Wertheimer, 1939) the carbohydrate utilization was decreased and gluconeogenesis increased. This was also demonstrated by Samuels, Reinecke & Ball (1942) on hypophysectomized rats and even on eviscerated preparations (Roberts, Samuels & Reinecke, 1944; Reinecke & Roberts, 1944). Most important are the experiments on isolated diaphragms of rats, reported by Lundbaek & Stevenson (1948) and by Gilmore & Samuels (1948) which, indicate that this muscle in a carbohydrate-fed rat utilizes glucose at twice the rate of that of the fat-fed animal when both are exposed to the same environment. It would appear, therefore, that the local cellular metabolic system is affected. A cellular adaptation to certain nutritional conditions must have occurred in this case by depressing carbohydrate metabolism. This shifting in cell metabolism from carbohydrate to fat utilization persists even in tissues isolated from the organism. The findings in these experiments cannot be due primarily to hormonal regulation, but seem to be brought about by some metabolic products in the cell itself, acting as cellular regulators whose effects are of long duration. Acetate and glycine are important metabolic products of the cell possibly responsible for this cellular regulation. This brings us to the assumption that the utilization of carbohydrates on the one side and of fats and proteins on the other side, is regulated primarily by the cell itself, certain metabolic products being responsible for this regulatory action. The action of hormones would then be secondary.

SUMMARY

1. More glycogen in liver and muscle is found after a 24 hr. fast in rats, which received 3–12 % acetate with their diet, than in their controls, where equivalent amounts of carbohydrate replaced the acetate. This was not the case after shorter fasting periods or after a lengthy fast of 72 hr.

2. Acetate-fed rats which received phlorrhizin during the 24 hr. fast showed the same low glycogen values as the controls 3 hr. after the phlorrhizin administration, but had much more liver glycogen than the controls 17 hr. after the phlorrhizin injection.

3. A similar effect on the fasting glycogen was not evinced by propionate, butyrate, stearate, lactate or citrate.

4. This *in vivo* action of acetate is discussed in connexion with the results found with acetate *in vitro*.

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The Swelling of Collagen in Alkaline Solutions

2. SWELLING IN SOLUTIONS OF UNIVALENT BASES

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In Part 1 (Bowes & Kenten, 1950) it was shown that changes in cohesion as well as osmotic effects must play an important part in determining the swelling of collagen in solutions of sodium hydroxide. It seemed probable from these results that the increased swelling at high pH values was due to a decrease in the cohesion of the collagen, and that this decrease in cohesion was associated with the presence of a high concentration of hydroxyl ions rather than with any specific effect of the sodium ion. It has been suggested, however, that bases differ in their effect on collagen; e.g., ammonia is reported to cause more splitting up of the fibres (an indication of decreased cohesion) than sodium hydroxide (Kaye & Jordan Lloyd, 1924).

The swelling of collagen in solutions of a number of strong and weak bases has, therefore, been studied to gain information regarding these effects, and, in particular, with respect to the nature of the decrease in cohesion occurring at high pH values in solutions of sodium hydroxide. In a brief account of the preliminary work (Bowes & Kenten, 1947) it was shown that, in contrast to strong bases, the swelling of collagen in solutions of weak bases decreased at high pH values in apparent accordance with the Donnan theory. It was suggested that the undissociated base in some way inhibited the action of the hydroxyl ions on cohesion. By comparison with swelling curves obtained in the presence of organic solvents it has now been shown that the decrease is related to the progressive departure from an aqueous medium as the concentration of base increases.

EXPERIMENTAL

Materials

Collagen. The alkali-treated sheepskin collagen D and E described in Part 1 (Bowes & Kenten, 1950) was used.

Guanidine. The free base was prepared from the sulphate by the addition of a slight excess of $Ba(OH)_2$ followed by removal of excess Ba^{++} by the addition of H_2SO_4. The solution so prepared gave no test for SO_4^{--}, and the ash content was 0·0012 g./100 ml.

Tetramethylammonium hydroxide. An aqueous solution of tetramethylammonium bromide prepared from trimethylamine and methyl bromide (Hickinbottom, 1948) was treated with Ag_2O, and the solution filtered. The resulting solution was 0·66 N.

Diethylamine. Technical diethylamine was shaken with charcoal, dried over solid NaOH and the free base distilled off.

The other bases and organic solvents were either of A.R. or technical grade as supplied by British Drug Houses Ltd.

Determination of swelling curves

The technique adopted was the same as that used in Part 1 (Bowes & Kenten, 1950). The weight of water or solution held by the collagen, expressed in terms of percentage of the moisture- and ash-free weight, was again taken as a measure of swelling. No special precautions were taken to avoid contamination with CO_2 or loss of base by volatilization, but the time during which the solutions were exposed to the atmosphere was kept to a minimum.

With the weak bases, changes in pH with concentration were small, and, in addition to pH measurements, the final concentration of base was determined by titration. In some cases it was obvious that appreciable amounts of collagen were dissolved, but except when using acetone and dioxan, it was not possible to check this by N determinations owing to the high N content of the bases. Where appreciable solution of the collagen was suspected, the samples were air-dried, heated in an air oven at 106° for 6 hr. and the percentage loss in weight, based on final weight, taken as a measure of the swelling. This method of calculating the results is not ideal, since appreciable amounts of base may be retained by the collagen after drying and heating under alkaline conditions may lead to some decomposition of the collagen.

All pH values were determined at 20° and pH values quoted refer to the values at 20°. The Cambridge glass electrode standardized with phthalate buffer (pH 4·00) and borate buffer (pH 9·22) was used for pH values below 9·5, and the special Beckman electrode standardized with Na_2CO_3-$Na_2B_4O_7$ buffer (pH 10·00) for the pH values above 9·5. There is some doubt regarding the pH values of the solutions containing high concentrations of weak bases or organic solvents. The pH values may be in error owing to the limitations of the glass electrode in alkaline solutions (Dole, 1941*a*). This error is probably small in solutions of organic bases (Dole, 1941*a*; Hill, 1929; Hubbard, Hamilton & Finn, 1939) and will cause the observed pH to be lower than the true value. McInnes & Dole (1930) found little error with 0·1 N solutions of tetramethylammonium hydroxide. Deviation of the activity of water from unity may also introduce errors, but by analogy with similar effects in acid solutions and solvent water mixtures these are also likely to be small (Dole, 1941*b*).

It is the hydroxyl-ion activity which governs swelling, however, and although the observed pH values may not be greatly in error, alterations in K_w and in the activity of water

will affect the pH-pOH relationship, and hence the position of the swelling curves relative to one another. For true comparison with the NaOH curves, those of the weak bases should be progressively shifted towards the left as the concentration of base increases. Judging from the values of K_w in dioxan and water mixtures (K_w decreases from $0{\cdot}6809 \times 10^{-14}$ to $1{\cdot}234 \times 10^{-16}$ as the concentration of dioxan increases from 0 to 45%; Harned & Fallon, 1939), and from the change in pH of 0·01 N-NaOH solutions on the addition of acetone (Table 1), this shift should be of the order of one pH unit when the concentration of base is increased from zero to between 30 and 40%.

RESULTS

Swelling curves in solutions of strong and weak univalent bases

The swelling curve of collagen in tetramethylammonium hydroxide solutions was almost identical with that in sodium hydroxide solutions (Fig. 1);

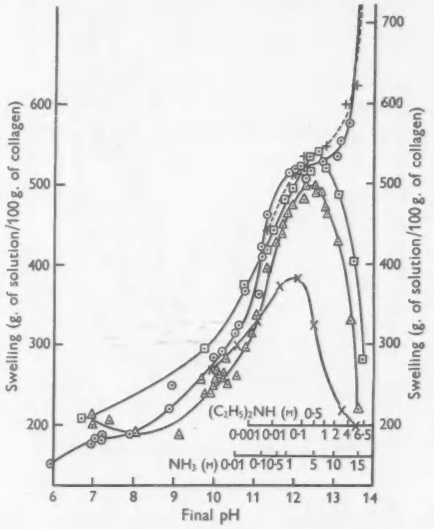
Swelling (g. of solution/100 g. of collagen)
Swelling (g. of solution/100 g. of collagen)
Final pH
$(C_2H_5)_2NH$ (M) 0·5
0·001 0·01 0·1 1 2 4 6·5
NH_3 (M) 0·01 0·1 0·5 1 5 10 15

Fig. 1. Swelling curves of collagen in solutions of NaOH, ⊙—⊙; tetramethylammonium hydroxide, +---+; diethylamine, ⊡—⊡; NH_3, △—△; NH_3 with the addition of 0·5 M-NaCl, ×—×.

swelling increased rapidly with pH up to 11·5, increased more slowly in the range 11·5–13·0, and then increased rapidly with further increases in pH. The curve for guanidine (Fig. 2) was of the same shape, but swelling was less than in sodium hydroxide solutions at pH values up to 13·5. The smaller amount of swelling at pH values below 13·5 is probably due to combination of guanidinium ions with the collagen so that they make no contribution to the swelling pressure.

The swelling curves of collagen in solutions of the weak bases (Figs. 1 and 2) varied only slightly from that in sodium hydroxide solutions up to about pH 12·5, but at higher pH values they began to show considerable divergencies, the swelling decreasing with pH in apparent agreement with the Donnan Theory, until at about pH 13·5 it approached that found in the isoelectric range. The decreased swelling is not due to disintegration of the collagen, since the pieces in the most concentrated solutions were obviously unswollen. Also on drying there was no appreciable loss in weight of the collagen. Although variations in the pH-pOH relationship with increasing concentration of base give rise to some doubt regarding the exact position of the curves for the weak bases in relation to the curve for sodium hydroxide (p. 524), such variations do not alter the fact that the swelling decreased with increasing concentration of base. The addition of 0·5 M-sodium chloride decreased swelling in solutions of ammonia at all pH values, but the shape of the curve was

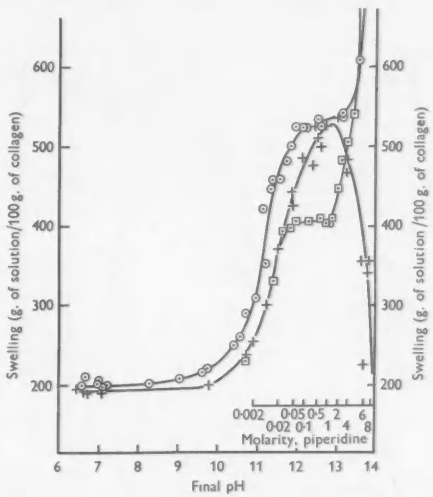
Swelling (g. of solution/100 g. of collagen)
Swelling (g. of solution/100 g. of collagen)
Final pH
0·002 0·05 0·5 2 6
0·02 0·1 1 4 8
Molarity, piperidine

Fig. 2. Swelling curves of collagen in solutions of NaOH, ⊙—⊙; guanidine, ⊡—⊡; piperidine, +—+.

unchanged. At pH 12·0 the decrease in swelling was of the same order as that in sodium hydroxide solutions at equivalent sodium chloride concentrations.

These findings rule out the possibility that the decrease in cohesion observed at high pH values in sodium hydroxide solutions is due to a specific effect of the sodium ion and tend to confirm the view that it is associated with high concentrations of hydroxyl ions which by some mechanism, probably breaking of hydrogen bonds, reduce the cohesion of the collagen. The reason why this effect is apparently

not operative in solutions of weak bases was not at first clear. The most likely explanation appeared to be that the undissociated base inhibited the effect of the hydroxyl ions on cohesion. If this were so, then it might be expected that the molar concentration of the base would be a major factor in determining the effect.

The effect of the concentration of organic base at high pH *values*

To test the suggestions made above, the swelling of collagen in sodium hydroxide solutions containing varying amounts of ammonia was determined. The ammonia concentration was kept constant at 3·8, 7·6 and 10·6N and the pH was varied by the addition of sodium hydroxide (Fig. 3). There was a considerable scatter of points, but the results indicated that at any given ammonia concentration, the amount of swelling was at first unaffected by rise of pH, but

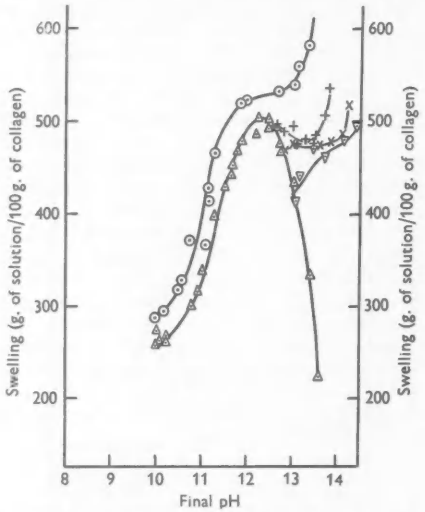

Fig. 3. The effect of additions of NH_3 on the swelling curve of collagen in NaOH solutions. NaOH, ⊙—⊙; NH_3, △—△; NaOH + 3·8M-NH_3, +—+; NaOH + 7·6M-NH_3, ×—×; NaOH + 10·6M-NH_3, ▽—▽.

finally increased in the same way as with sodium hydroxide alone. Apparently, the higher the pH, the greater the concentration of weak base necessary to suppress swelling. Consideration of the curves in Figs. 1–3 indicates that at equivalent molar concentrations diethylamine was much more effective than ammonia, and slightly more effective than piperidine in suppressing swelling. The effect of a number of different bases at the same molar concentration over a range of pH values was, therefore, determined, using 5M-solutions of the bases, adjusted to the required pH's with sodium hydroxide or hydrochloric acid. With all the bases swelling increased with pH, but the relative effects varied greatly (Fig. 4). There was an indication that the effectiveness of the bases in suppressing swelling was related to their concentration in g., but some specific effect was also apparent; e.g. diethylamine had a much greater effect than other bases at the same or greater concentrations. It was also found that the swelling curve in 36% (v/v) acetone solutions was not very different from that found with pyridine and some of the other weak bases (Fig. 4).

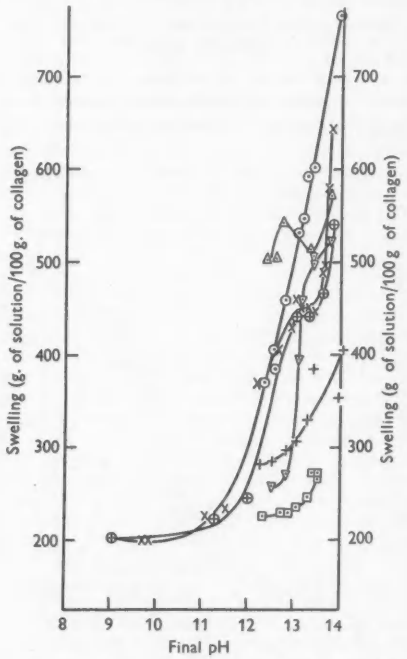

Fig. 4. The swelling of collagen in 5M-solutions of NH_3, △—△; monomethylamine, ⊙—⊙; dimethylamine, ▽—▽; diethylamine, ⊡—⊡; pyridine, ×—×; piperidine, +—+; acetone, ⊕—⊕.

The effect of acetone and dioxan on swelling

To obtain further information on the influence of the medium on swelling, the effect of increasing concentrations of acetone and dioxan on the pH and the swelling properties of 0·01 and 0·1N-sodium hydroxide solutions was determined. The addition of acetone progressively increased the pH (Table 1) due to alterations in K_w (p. 525), the shift in pH being of the order of 1·1–1·5 units in 50% (v/v) acetone. The shift in pH with increasing dioxan concentration was much less, about 0·7 pH unit; this may have

Table 1. *The effect of acetone and dioxan on the* pH *values of sodium hydroxide solutions and the effect of acetone on the soluble nitrogen derived from collagen after soaking for* 3 *days in sodium hydroxide solutions at* 20°

(The time elapsing between the addition of the dioxan and the determination of pH varied from 0·5 to 1 hr.)

	Acetone in 0·01 N-NaOH			Acetone in 0·1 N-NaOH			Dioxan in 0·01 N-NaOH	
	pH			pH			pH	
Concentration of acetone or dioxan (ml./100 ml.)	Initial	After 3 days' contact with collagen	Soluble N (mg./g. collagen)	Initial	After 3 days' contact with collagen	Soluble N (mg./g. collagen)	Initial	After 3 days' contact with collagen
0	12·05	11·98	3·8	12·97	12·99	9·2	12·06	12·02
5	—	12·10	2·3	13·09	13·09	9·4	12·08	12·05
10	12·28	12·21	6·1	13·18	13·19	10·5	12·22	12·08
15	12·38	12·34	4·2	13·36	13·33	7·8	12·12	12·10
20	—	12·45	3·8	13·44	13·47	9·1	12·28	12·12
25	12·68	12·58	5·1	13·56	13·59	9·6	12·38	12·13
30	12·75	12·72	12·2	13·67	13·67	13·0	12·46	12·10
35	12·99	12·86	9·0	13·78	13·81	14·3	12·50	12·06
40	13·12	13·02	9·3	13·97	13·92	16·5	12·60	11·93
45	13·31	13·20	5·4	14·06	14·01	15·8	12·72	11·62
50	13·48	13·36	7·1	14·13	14·15	16·4	12·74	11·12

been due to reaction with the dioxan since after 3 days the pH of the concentrated dioxan solutions had fallen below that of the original sodium hydroxide (Table 1).

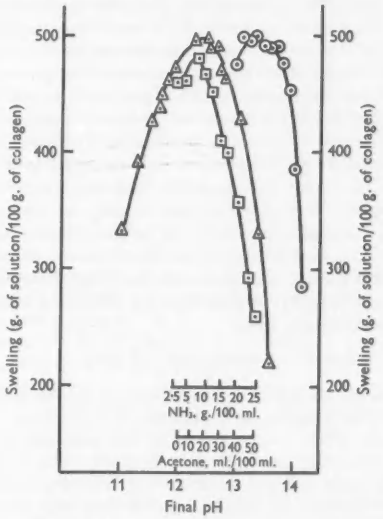

Fig. 5. The effect of increasing additions of acetone on the swelling of collagen in 0·01 (⊡—⊡) and 0·1 M-NaOH (⊙—⊙) solutions. The swelling curve obtained with NH_3 (△—△) is included for comparison.

The swelling of the collagen decreased as the concentration of the acetone increased and in 50 % (v/v) acetone the swelling was of the same order as in the isoelectric range. With acetone in 0·01 N-sodium hydroxide the net result of these two effects, rise in pH and decrease in swelling, was to give a swelling curve similar in shape to those found with the weak bases (Fig. 5) except that it descended more sharply with pH, since there was no increase in hydroxyl ion concentration due to dissociation of base. With 0·1 N-sodium hydroxide the curve was of the same shape,

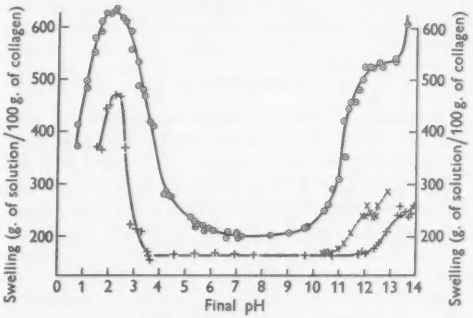

Fig. 6. The effect of the addition of 50 % (v/v) acetone on the swelling of collagen in HCl and NaOH. No acetone, ⊙—⊙; 50 % acetone, +—+, and 50 % acetone, allowance made for effect on K_w, × —— ×.

but was shifted to correspondingly higher pH values. Acetone also decreased swelling in the isoelectric range and in acid solutions (Fig. 6).

The effect of organic solvents on solution of collagen

Although acetone and dioxan decreased swelling in alkaline solutions, they also tended to favour solution of the collagen (Tables 1 and 2). Other

organic solvents miscible with water, e.g. *iso*propanol and pyridine, also had a similar effect (Table 2). If the hydroxyl ion concentration exceeded about 0·5 M the above solvents at 5 M concentrations and 20° caused almost complete solution of the collagen in 3 days.

Table 2. *The effect of organic solvents on the amount of collagen dissolved during 3 days' soaking in sodium hydroxide solutions at 20°*

Solvent	Normality NaOH	pH at 20°	Percentage loss in weight
Dioxan (40%, v/v)	0·79	Approx. 14·1	Nearly all dissolved
	0·53	Approx. 14·1	Nearly all dissolved
	0·26	Approx. 14·1	All dissolved except grain
n-Propanol (37%, v/v)	0·63	13·9	Dissolved in 1 day
	0·30	13·7	Dissolved in 2 days
Pyridine (40%, v/v)	0·10	13·8	80
	0·08	13·7	56
	0·05	13·7	14
	0·04	13·6	8
	0·02	13·4	2
Diethylamine (40%, v/v)	0·002	13·5	2
Acetone (36%, v/v)	0·63	14·1	Dissolved in 1 day
	0·30	14·0	Dissolved in 2 days
Acetone (50%, v/v)	0·05	13·9	3·5
	0·03	13·8	2·2
	0·02	13·7	2·7
	0·015	13·6	2·5
	0·01	13·4	2·4
	0·007	13·3	2·0
	0·002	12·6	0·9

DISCUSSION

The results of this investigation confirm the suggestion that the increased swelling at high pH values in sodium hydroxide solutions is associated with the presence of a high concentration of hydroxyl ions rather than with any specific effect of the sodium ion. The other strong bases studied gave swelling curves of the same shape as those obtained with sodium hydroxide and the addition of 0·5 M-sodium chloride did not alter the shape of the ammonia curve, but reduced swelling at all pH values. Variations in swelling caused by the different bases, both strong and weak, at pH values up to 12·5 can be attributed to variations in the extent to which the cations combine with collagen, thus reducing their contribution to the swelling pressure. On this basis the guanidinium cation has a much greater, and the ammonium ion a slightly greater, affinity for collagen than the cations of the other bases studied. The power of guanidine salts to denature proteins may be related to the high affinity of the guanidinium ions for proteins. The decrease in swelling at high pH values in solutions of weak bases, in apparent agreement with the Donnan theory, is less readily explained. It was thought at first that the presence of the undissociated base in some way inhibited the effect of the hydroxyl ions on cohesion (Bowes & Kenten, 1947), but since depression of the swelling was found to be dependent on the percentage rather than on the molar concentration of the base it seemed more likely that it was related to changes in the medium. The fact that swelling curves of similar shape are obtained by progressively increasing the concentration of acetone in 0·01 and 0·1 N-sodium hydroxide solutions lends support to this view. However, there are also specific differences between the bases, for the extent to which swelling is decreased is not entirely dependent on the percentage composition of their solutions; e.g. swelling in diethylamine solutions is much less than that in solutions of other bases at similar or higher percentage concentrations.

The decrease in swelling with increasing concentration of weak base or organic solvent must be due either to a lowering of the swelling pressure or to an increase in cohesion. Since the presence of organic solvents tended to disperse collagen, which is in itself an indication of decreased cohesion, the former suggestion seems the more likely. Some effect analogous to the 'salting out' of soluble proteins may also play a part as the effects are apparent even when the swelling pressure is very low, e.g. at very high or low pH values and in the isoelectric range.

The mechanism by which the swelling pressure is decreased is not at present clear. It is unlikely, judging from the titration curves of proteins in organic solvents, and in particular of gelatin (Lichtenstein, 1940), that it can be attributed to alterations in the ionization of the reactive groups of the collagen. It is possible that it may be connected with the effect of the solvents on the activities of the ions. The distribution of the ions between the internal and the external phases in the Donnan equilibria is more properly represented by activities than by concentrations. Thus

$$e' = 2a_y y + a_z z - 2a_x x,$$

where e', x, y and z have their usual significance (Bowes & Kenten, 1950; Bolam, 1932) and a_x, a_y and a_z are their respective activity coefficients. In aqueous sodium hydroxide solutions the activity coefficients will not differ greatly from unity, but in the presence of organic substances the activity coefficients will be appreciably lower, and e', and hence the swelling pressure, will be proportionately smaller. To what extent the swelling may be affected by differences between the activity of the components of the solvent phase inside and outside the fibres is not evident.

Although giving no positive evidence regarding the possible reasons for the decrease in cohesion of

the collagen at high pH values, the results are not inconsistent with the view that the main cause is breaking of intermolecular links between the polypeptide molecules. The breaking of hydrogen bonds between polypeptide groups will almost certainly be involved to some extent, but in view of recent work by Lindley (1947), who finds that the specific removal of amide groups greatly increases the solubility of wool in alkaline solutions, links involving these groups may also be concerned. Treatment of collagen in sodium hydroxide solution at pH 13·7 for 3 days decreased the amide nitrogen from 0·36 to 0·12 mmol./g. and caused over 700 % swelling, whereas treatment in ammonia or diethylamine solutions at the same observed pH values had no appreciable effect on the amide nitrogen and caused no swelling. In this connexion it is of interest that Ames (1944) states that loss of amide nitrogen is essential for the production of gelatin from collagen. Another possibility is that the decrease in cohesion is related to removal of mucopolysaccharides. Partridge (1948), as a result of work on the state of combination of chondroitin sulphate in cartilage, suggests that the chondroitin sulphate, acting as a multivalent anion, cements together the protein molecules to form fibrous macromolecules. He finds that breakdown of the gelatin-chondroitin sulphate complex causes a large decrease in the viscosity of gelatin. Treatment with alkali or calcium chloride are common methods of extracting chondroitin sulphate from cartilage and other tissues, and since both these treatments lead to a decrease in cohesion (for information on the effect of calcium chloride see Bowes, 1950) it seems possible that its removal may at least be a contributory cause of this decrease.

SUMMARY

1. The swelling of ox-hide collagen in solutions of strong and weak bases has been studied. Differences in swelling at pH values up to pH 12·5 are ascribed to variations in the affinity of the cations for the collagen.

2. The swelling curves obtained with tetramethylammonium hydroxide and guanidine were of the same general shape as the curve obtained with sodium hydroxide, suggesting that the increased swelling at high pH values is due to increase in the hydroxyl ion concentration rather than to any specific effect of the sodium ion.

3. With weak bases swelling decreased at pH values above 12·5, in apparent accordance with the Donnan theory. Curves of similar shape were obtained by the addition of increasing amounts of neutral organic solvent, and it is concluded that the decrease in swelling is due to the progressive departure from an aqueous medium. There were also specific effects due to the different bases.

4. The effect of the organic solvents was presumably due to decrease in the swelling pressure rather than to increase in cohesion, since their presence was found to favour solution of the collagen.

5. The results are consistent with the view that decrease in the cohesion of collagen at high pH values is due to breaking of intermolecular linkages. The possible nature of these linkages is discussed.

The authors wish to thank Prof. C. W. Davies and Dr D. H. Everett for advice on the interpretation of pH measurements in solutions of weak bases and organic solvents, and the Council of The British Leather Manufacturers' Research Association for permission to publish this paper.

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The Swelling of Collagen in Alkaline Solutions

3. SWELLING IN SOLUTIONS OF BIVALENT BASES

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The swelling of collagen in solutions of a number of univalent bases has been described in previous communications (Bowes & Kenten, 1950*a*, *b*). In the present paper the work has been extended to cover the bivalent bases, calcium, strontium and barium hydroxide.

EXPERIMENTAL

The raw material and technique were the same as those used in Parts 1 and 2 (Bowes & Kenten, 1950*a*, *b*). Saturated solutions of $Ca(OH)_2$, $Sr(OH)_2$ and $Ba(OH)_2$ were prepared by shaking excess of the solid hydroxide with boiled distilled water and allowing to settle.

RESULTS

Swelling in solutions of calcium, strontium and barium hydroxides

The swelling of collagen in solutions of calcium, strontium and barium hydroxides was considerably less than in sodium hydroxide solutions at all pH values, and more especially in the range 11·0–13·0 (Fig. 1). The curves were of the same general shape as the sodium hydroxide curve, showing a flattening between pH 12·0 and 13·0 followed by a sharp increase in swelling at higher pH values. Appreciable swelling commenced only at about pH 11, i.e. approximately one pH unit higher than with sodium hydroxide. The amount of swelling decreased in the order barium, strontium, calcium hydroxide. Even in barium hydroxide solutions the swelling, in excess of that occurring in the isoelectric range, was barely half that in sodium hydroxide solutions, that in strontium hydroxide was rather less and that in calcium hydroxide was only about one-eighth of that in sodium hydroxide solutions.

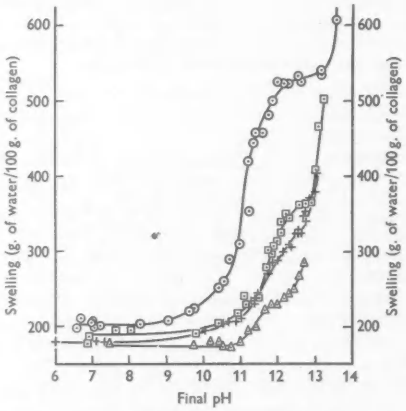

Fig. 1. Swelling of collagen in solutions of $Ca(OH)_2$, △—△; $Sr(OH)_2$, +—+; $Ba(OH)_2$, □—□. Curve ⊙—⊙ for NaOH solutions included for comparison.

The effect of calcium and sodium chlorides on swelling in calcium hydroxide solutions

The addition of calcium chloride increased the swelling in calcium hydroxide solutions at all pH values (Fig. 2), the presence of 0·09 M-calcium chloride slightly increased the swelling, and as the concentration was further increased the swelling progressively increased until in the presence of 1·0 M-calcium chloride it was more than twice that in the hydroxide alone. The addition of a salt must decrease the swelling pressure, and the fact that calcium chloride increased swelling indicates that it must have a very marked effect in decreasing the cohesion of the collagen.

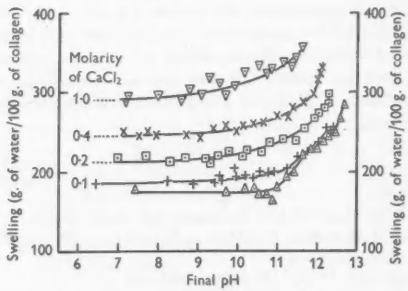

Fig. 2. The effect of addition of $CaCl_2$ on the swelling of collagen in $Ca(OH)_2$ solutions (△—△, no $CaCl_2$).

The addition of sodium chloride also increased swelling in calcium hydroxide solutions (Figs. 3 and 4). As low a concentration as 0·001 M-sodium chloride

caused a small increase in swelling, and with 0·02 M-sodium chloride the swelling was approximately doubled. The swelling continued to increase until the concentration reached 0·1 M and then remained approximately constant as the concentration was further increased to 0·2, 0·5 and 1 M. Above 0·1 M

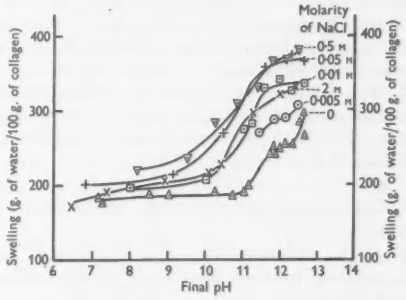

Fig. 3. The effect of addition of NaCl on the swelling of collagen in $Ca(OH)_2$ solutions.

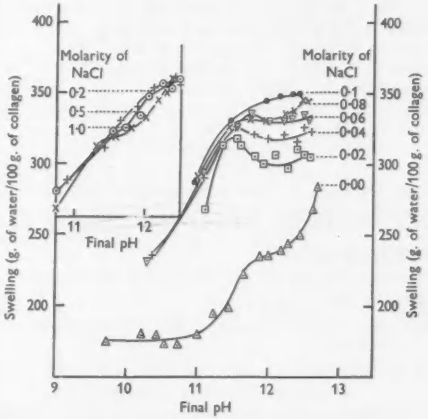

Fig. 4. The effect of addition of NaCl on the swelling of collagen in $Ca(OH)_2$ solutions.

the shape of the curve began to change, the flattening in the pH range 12·0–12·5 was less marked and the curve tended to descend more sharply with decrease in pH until it became concave in shape. Similar changes in the shape of the swelling curves in sodium hydroxide solutions with increasing sodium chloride concentration have already been described (Bowes & Kenten, 1950*a*).

The maximum shown in the neighbourhood of pH 11·5 with 0·02 and 0·04 M-sodium chloride may be due to changes in the calcium-ion concentration. A change in pH from 11·5 to 12·5 involves a tenfold change in the calcium-ion concentration, and since the sodium-ion concentration is constant and low, it is possible that such a rise may increase the Ca^{++}/Na^{+} ratio sufficiently to cause a decrease in the swelling.

DISCUSSION

On the basis of the Donnan theory of membrane equilibria it might be expected that the swelling pressure and hence the amount of swelling would be the same with all three alkaline earth bases, and rather more than half that in sodium hydroxide solutions at corresponding pH values, the ratio of the membrane potentials in uni- and bi-valent systems being 0·66 (Bolam, 1932). Only with barium hydroxide, however, did the swelling approach half that found with sodium hydroxide and with calcium hydroxide it was very much less. The low amount of swelling in calcium hydroxide is the more surprising when it is remembered that the second dissociation constant of calcium hydroxide is comparatively low, 0·031 (Davies, 1938), so that about one-third of the calcium functions as a monovalent cation, whereas with barium hydroxide the corresponding dissociation constant is 0·23 (Davies, 1939) and nearly all the barium will be present as the divalent cation.

The unexpectedly low swelling in solutions of bivalent bases, especially calcium hydroxide, may be due to their having a smaller effect on the cohesion than sodium hydroxide, or to incomplete ionization of the protein salts. In view of the relative effects of calcium and sodium chlorides on swelling in calcium hydroxide (Fig. 2), and in sodium hydroxide (Bowes & Kenten, 1950*a*), respectively, and of the lowering of the shrinkage temperature caused by calcium chloride (Theis & Steinhardt, 1942), it would appear that the calcium ion reduces the cohesion of the protein to a much greater extent than the sodium ion and the first suggestion is, therefore, improbable. There is a considerable amount of evidence pointing to the combination of alkaline earth cations, especially calcium, with proteins (Greenberg, 1944) and by analogy with the dissociation constants of calcium and barium salts of the hydroxy and dicarboxylic acids (Cannan & Kibrick, 1938), calcium is likely to have a greater affinity than barium for proteins. The present observations suggest that the affinity of calcium, strontium and barium for collagen decreases in the order $Ca > Sr \geqslant Ba$. No evidence is at present available regarding the relative effect of these three cations on cohesion.

It seems that three factors participate in determining the effect of additions of sodium chloride to solutions of calcium hydroxide (or of any univalent salt to a solution of a bivalent base) on the swelling of collagen: (1) The effect of the univalent cation in increasing the swelling pressure. (2) The effect of

the salt in decreasing the swelling pressure. (3) The effect of the salt on cohesion. The curves shown in Fig. 3 suggest that at low concentrations of sodium chloride, increase in the sodium-ion concentration is the predominant factor, the rise in the Na^+/Ca^{++} ratio increasing the swelling pressure with the result that swelling increases. As the concentration of sodium chloride increases, the effect of salt in depressing the swelling pressure becomes apparent, and balances the effect of increase in the sodium-ion concentration, so that the swelling remains constant. The change in the shape of the curve above 0·1M suggests that cohesion is beginning to be affected; at concentrations of this order the swelling pressure probably begins to decrease, but the decrease in cohesion balances this and the swelling remains unchanged. At concentrations of 2M or greater, sodium chloride begins to decrease the swelling again, probably by an action similar to that occurring in the salting out of proteins (Cohn & Edsall, 1943).

SUMMARY

1. The swelling of collagen in solutions of bivalent bases was found to decrease in the order barium hydroxide ≥ strontium hydroxide > calcium hydroxide. Swelling in barium hydroxide solutions was rather less than half, and that in calcium hydroxide solutions about one-eighth of that in sodium hydroxide solutions. The small amount of swelling in calcium hydroxide solutions is attributed to combination of calcium ions with the collagen.

2. The addition of calcium or sodium chlorides increased swelling in calcium hydroxide solutions. The increase with calcium chloride is attributed to decrease in the cohesion of the collagen, and the increase with sodium chloride to the addition of a univalent cation and consequent increase in swelling pressure.

The author thanks the Council of the British Leather Manufacturers' Research Association for permission to publish this paper.

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The Separation of the C_{12}-C_{18} Fatty Acids by Reversed-phase Partition Chromatography

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Work in this laboratory (Pollock, Howard & Boughton, 1949) on inhibition and promotion of bacterial growth by fatty acids revealed the need for analytical and preparative methods adapted to handling small quantities of these substances. Chromatography seemed to offer a possible solution to these problems.

Early attempts at adsorption chromatography showed little promise until Claesson (1946) and Holman & Hagdahl (1948, 1949) developed frontal and displacement analysis methods. These, though very successful, require rather elaborate apparatus and larger amounts of material than we wished to work with. We turned, therefore, to partition chromatography.

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This method had already been successfully applied to the separation of the lower fatty acids (Elsden, 1946; Ramsey & Patterson, 1945; Peterson & Johnson, 1948; Moyle, Baldwin & Scarisbrick, 1948). Ramsey & Patterson (1948) have extended the method to the longer chain (C_{11}-C_{19}) acids using 2-aminopyridine and furfuryl alcohol as their solvent system. They were able to separate acids differing by two CH_2 groups. It seems improbable that suitable solvent systems of this type could be found for much longer-chain fatty acids or for lipophilic substances lacking acidic groups. These substances would have, in general, partition coefficients too much in favour of the less polar phase. We tried, therefore, to evolve a partition chromatogram in which the less polar phase is stationary. The choice of solvent systems

should then be very much more extensive and the method correspondingly more flexible.

Attempts have previously been made to reverse the phases. Boscott (1947) suggested the use of cellulose acetate, and Synge (1949) attempted to apply this to the separation of peptides which were too fast running for the usual type of chromatogram, but with disappointing results. Boldingh (1948) successfully separated methyl esters of long-chain fatty acids on filter paper impregnated with vulcanized rubber latex. He located the ester spots by dyeing with Sudan III. This was not very satisfactory, nor was the method well adapted for preparative work.

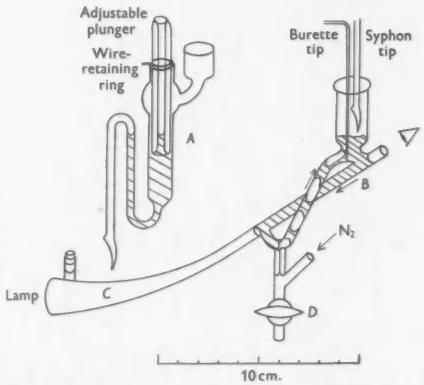

Fig. 1. Diagram of apparatus used. *A*, adjustable siphon; *B*, titration cell; *C*, tube filled with $CHCl_3$ to transmit light to titration cell; *D*, tap to empty cell.

The problem of finding a hydrophobic support for the less polar phase of a solvent system was solved by treating kieselguhr with dichlorodimethylsilane vapour and thus rendering it 'unwettable' by strongly polar solvents (cf. Martin, 1949). This treated kieselguhr successfully retains the less polar phase of numerous solvent systems, e.g. it will retain the acetone phase of an acetone-aqueous ammonium sulphate mixture and the paraffin phase of a paraffin-aqueous acetone mixture. The stability of the columns made with this support depends upon the interfacial tension of the two phases. When this is high stable columns can be made from treated kieselguhr holding as much as 1 ml. of stationary phase/g. The most suitable systems for use with fatty-acid mixtures were found to be aqueous methanol-octane and aqueous acetone-medicinal paraffin. The latter gives a very stable column which can be used repeatedly without losing the stationary phase. Using these systems, the straight-chain saturated acids from lauric to stearic have been successfully separated. Unsaturated and substituted acids behave similarly to the saturated acids. Only acids with an even number of carbon atoms have been studied.

METHODS

Preparation of kieselguhr. 'Hyflo Super Cel', obtained from British Drug Houses Ltd., was dried at 110° and, when cool, was allowed to stand in a desiccator containing dichlorodimethylsilane or stirred gently, whilst passing through it a stream of dry air previously drawn through the silane

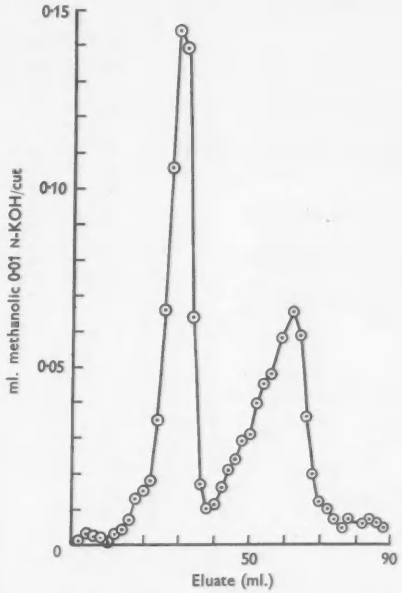

Fig. 2. The separation of a mixture of myristic and palmitic acids. Stationary phase, liquid paraffin saturated with 70% (v/v) aqueous methanol, moving phase, 70% (v/v) aqueous methanol saturated with liquid paraffin. Ambient temperature 35°.

derivative. The treated material should all float when shaken with water. The treated kieselguhr was washed with methanol until the washings were no longer acid to bromthymol blue, dried at 110° and stored for use.

Solvents. Solvents were normally used without special purification. Samples of alcohols with high acidities were distilled from solid KOH.

Columns. The standard columns used were 12 mm. in diameter. The mixture required for preparing the more polar phase, e.g. 60% (v/v) acetone in water, was equilibrated with the less polar solvent e.g. liquid paraffin. The relative proportions of the two phases equilibrated seemed to be immaterial. After equilibration, the two immiscible phases were allowed to separate and drawn off for use when required. At first, columns were packed with 4 g. of kieselguhr and 3·8–4·0 ml. of the less polar phase, but better results were

Table 1. *Behaviour of fatty acids in reversed-phase partition columns*

(The numbers indicate the start, peak and end of the bands as the total volume (ml.) of eluate collected at these points. These figures were obtained with the standard column 20 cm. long and 12 mm. in diameter made in the way indicated in the text with the solvent system quoted in the left-hand column. Solvent systems are defined as % (v/v) of the first-named substance in aqueous solution equilibrated with the second-named substance.)

Solvent system	Lauric acid			Myristic acid			Palmitic acid			Stearic acid			Oleic acid			Dihydroxy-stearic acid			Elaidic acid		
80% Methanol-*cyclo*-hexane	—	—	—	34	48	60	70	90	110	—	—	—	—	—	—	—	—	—	—	—	—
	—	—	—	28	40	52	60	78	100	—	—	—	—	—	—	—	—	—	—	—	—
60% Methanol-*n*-octane	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	10	15	24	—	—	—
70% Methanol-*n*-octane	22	34	44	48	70	80	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	22	32	40	48	68	78	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	—	—	—	52	76	94	110	192	220	—	—	—	—	—	—	—	—	—	—	—	—
80% Methanol-*n*-octane	—	—	—	24	32	40	42	56	78	90	116	130	—	—	—	—	—	—	—	—	—
	—	—	—	24	36	44	50	64	74	—	—	—	—	—	—	—	—	—	—	—	—
50% Acetone-medicinal paraffin	14	34	48	36	78	94	—	—	—	—	—	—	—	—	—	8	12	30	—	—	—
55% Acetone-medicinal paraffin	20	32	44	48	60	68	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	14	26	32	32	44	60	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	—	—	—	32	46	54	68	96	110	—	—	—	—	—	—	—	—	—	—	—	—
60% Acetone-medicinal paraffin	—	—	—	30	40	52	62	80	90	—	—	—	—	—	—	—	—	—	—	—	—
	—	—	—	20	40	50	50	80	96	—	—	—	—	—	—	—	—	—	—	—	—
65% Acetone-medicinal paraffin	—	—	—	10	24	45	32	52	70	—	—	—	—	—	—	—	—	—	—	—	—
	—	—	—	16	28	44	—	—	—	—	—	—	34	54	70	—	—	—	40	56	90
70% Acetone-medicinal paraffin	—	—	—	—	—	—	—	—	—	40	60	76	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	—	36	58	72	—	—	—	—	—	—	—	—	—

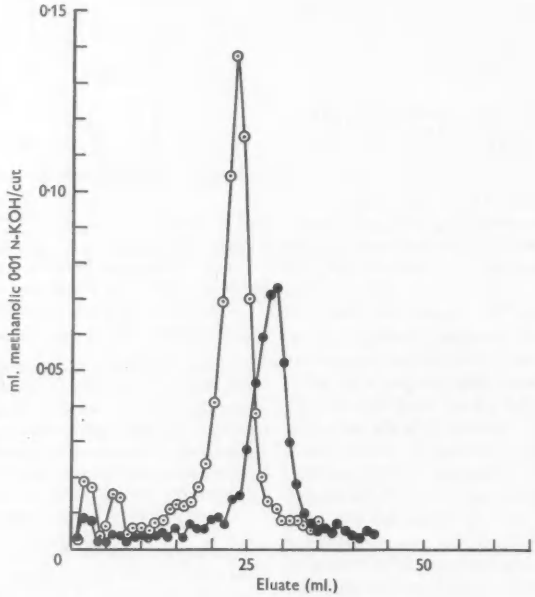

Fig. 3. Behaviour of single acids on reversed-phase partition column. ●, synthetic stearic acid; solvent system: 70% (v/v) aqueous acetone-liquid paraffin (b.p.). ⊙, synthetic myristic acid; solvent system: 60% (v/v) aqueous acetone-liquid paraffin (b.p.). Ambient temperature 35°.

obtained by using 9 g. of kieselguhr and 8 ml. of the less polar phase, giving a column 20 cm. long. The stated amounts of kieselguhr and less polar phase were stirred together in a beaker until a homogeneous powder was obtained; this was suspended in the more polar phase and the mixture was stirred by hand to remove lumps. The resulting slurry could not be packed into a column by gravity, but good columns were obtained by using a plunger. This was a perforated stainless steel disk attached at its centre to a long stainless steel rod. A portion of the stirred slurry was poured into the chromatograph tube and homogenized by rapid up and down movements of the plunger, which should fit the tube closely. The resulting fine suspension was then packed by slowly moving the plunger downwards. This procedure was repeated until all the material was packed. When the interfacial tension of the phases is low, packing cannot be effected by the plunger until the suspension has settled somewhat. It was found necessary to homogenize the kieselguhr-paraffin mixtures in a separate tube before starting to pack the column, and this was desirable on all occasions when the material was particularly lumpy. If air is inadvertently included during homogenizing, it must be removed by cautious evacuation prior to packing. Some of the solvent systems tested gave columns which tended to lose some of the stationary phase during use; aqueous methanol-$CHCl_3$ columns were particularly unstable, whereas aqueous acetone-medicinal paraffin columns have been used 14 times without changing their characteristics. The fluctuation of the room temperature during 24 hr. may so change the mutual solubilities of the less stable systems that the characteristics of the column alter. It is then advisable to maintain the column at a constant temperature if it is to be kept overnight. A constant temperature of 35° was normally used for convenience and because the increased solubility of fatty acids at that temperature was an additional advantage. There is no reason to think that other temperatures are

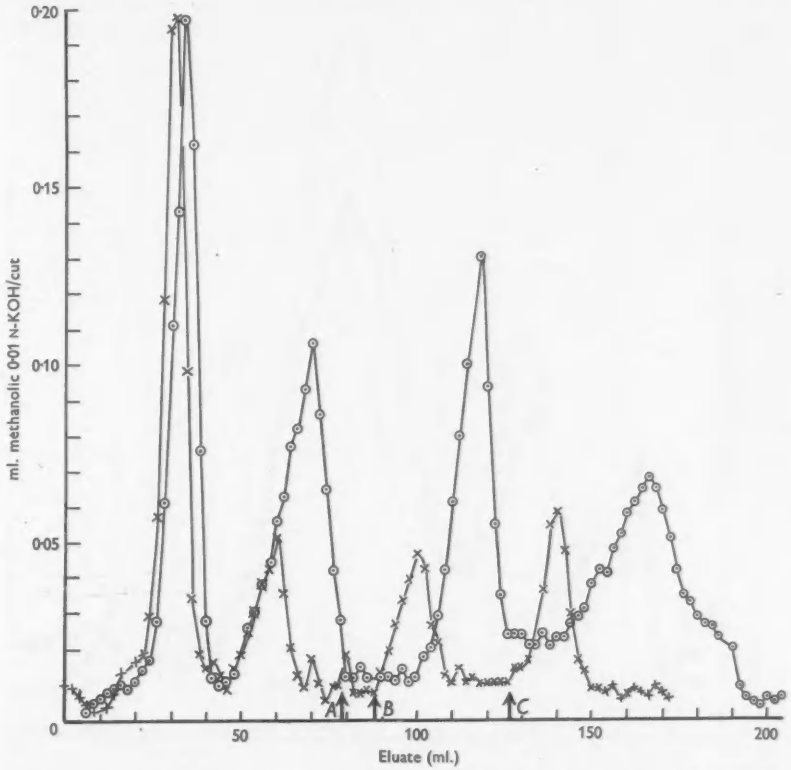

Fig. 4. The separation of a mixture of lauric, myristic, palmitic and stearic acids. ⊙, using 70 and 80% (v/v) aqueous methanol saturated with *n*-octane as the moving phase and *n*-octane saturated with 70% (v/v) aqueous methanol as the stationary phase. Moving phase changed from 70 to 80% aqueous methanol-*n*-octane at *B*. ×, using 55, 68 and 70% (v/v) aqueous acetone saturated with liquid paraffin (B.P.) as the moving phase and liquid paraffin saturated with 55% (v/v) aqueous acetone as the stationary phase. Moving phase changed from 55 to 68% aqueous acetone at *A* and to 70% aqueous acetone at *C*. Ambient temperature 35°.

unsuitable since good results have been obtained at room temperature when the column was used for a few hours only. The solvents were run down the columns at the rate of 20–50 ml./hr., but slower rates, down to 10% of this speed, give better separations. Such a slow rate is not, however, necessary in most cases.

Loading. The acids to be analysed were dissolved in the solvent to be used for development and the solution (approx. 2 mg. of each individual acid in approx. 2 ml. of solvent) was added to the top of the column. This solution was allowed to soak in and was washed down with two 2 ml. portions of the solvent before attaching the separating funnel as in Fig. 1. Fatty acids readily form supersaturated solutions. If these crystallize when loaded on to the column, the solid acid dissolves slowly and causes a steady trickle of acid down the column. High blanks and low recoveries result. When this crystallization is avoided, the recovery is within the range of 95–100%. If a mixture of fatty acids of very different chain length is encountered, it may not be possible to dissolve it in a solvent suitable for separating the shorter-chain acids. In this case a solvent suitable for the longer-chain acids must be employed and the first runnings from the column must be refractionated on the appropriate column.

Siphon. The siphon *A* (Fig. 1) may be adjusted to deliver various volumes by moving the plunger; 2 ml. was normally delivered.

Titrations. Titrations were carried out under N_2 using cresol red or, in later experiments, bromthymol blue as indicator. It was found convenient to dissolve the indicator in the developing solvent. Standardized 0·01 N-KOH in methanol was used for the titrations and stored in a dark internally waxed bottle. A 2 ml. microburette (Fig. 1), graduated by 0·01 ml., was used to deliver the alkali to the titration cup. The alkali was protected at all points from atmospheric CO_2. The accuracy and speed of the titrations was improved by using the apparatus shown in Fig. 1. Light from the lamp is led into the titration cell (*B*) by a tube (*C*) filled with $CHCl_3$, and the colour is observed by looking endwise down the cell.

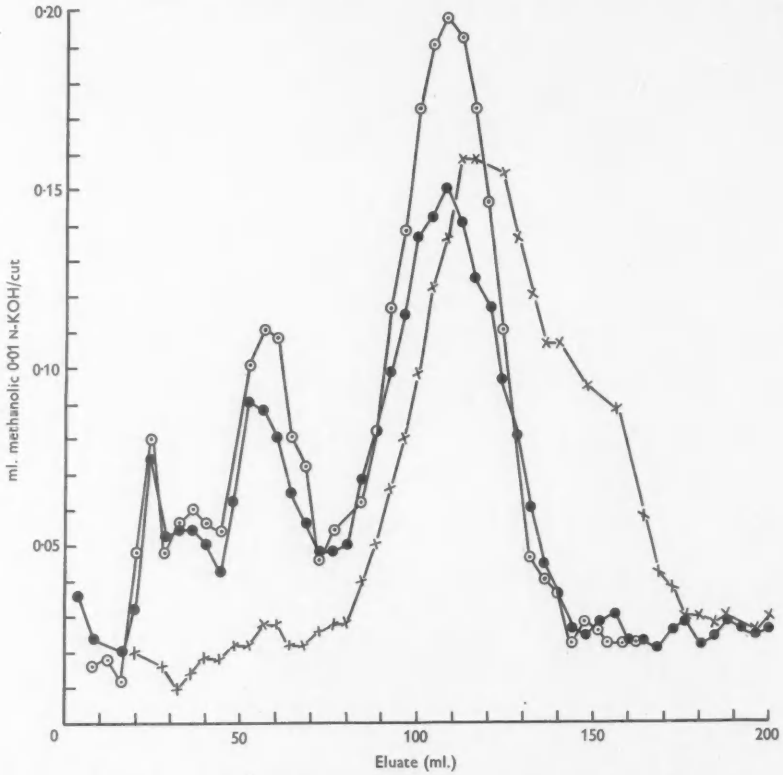

Fig. 5. Chromatographic behaviour of a commercial 'purified' oleic acid and its derivatives. ⊙, oleic acid (commercial 'purified'); ●, bromination product of above oleic acid; ×, elaidic acid prepared from commercial oleic acid. Ambient temperature 35°. Stationary phase, liquid paraffin (B.P.) saturated with 65% (v/v) aqueous acetone; moving phase, 65% (v/v) aqueous acetone saturated with liquid paraffin.

A capillary tube at the lowest part of the cell admits a nitrogen stream to circulate the liquid during the titration, and drains the cell when the tap (*D*) is opened. With this apparatus the colour change in a 6·5 cm. depth of liquid can be observed with a total volume of 2 ml. The approach of the end point is heralded by a flickering colour change. With bromthymol blue, the end point was taken to be the first persistent green colour. Attempts were made to follow the behaviour of the columns by a conductimetric method, but were abandoned because of erratic behaviour of the electrodes and the satisfactory nature of the titration method.

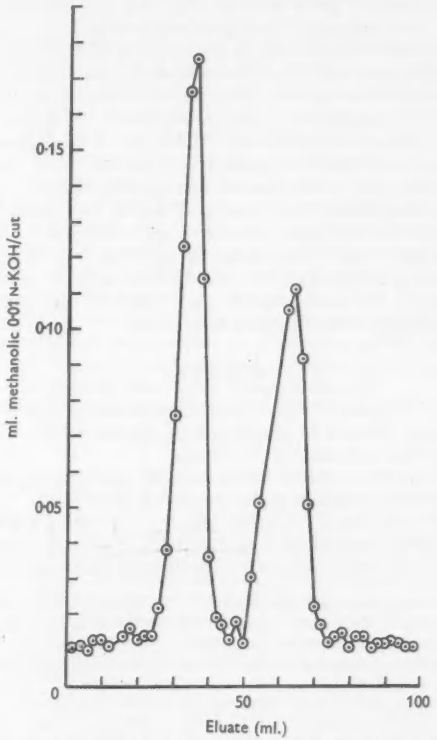

Fig. 6. The separation of a mixture of myristic and palmitic acids. Moving phase, 80% (v/v) aqueous methanol saturated with *n*-octane; stationary phase, *n*-octane saturated with 80% (v/v) aqueous methanol. Ambient temperature 35°.

Solvent systems. The most satisfactory systems tested were aqueous acetone-medicinal paraffin, and aqueous methanol-*n*-octane or *cyclo*hexane. The proportion of water in the solvent was chosen to give the most rapid development consistent with good separation. Increasing the proportion of water decreased the rate of development and improved the separation, but increased the width of the band. The most satisfactory procedure with a complicated mixture of acids was to change the solvent after the shorter-chain acids had emerged. The change was to a solvent containing a smaller proportion of water. The points at which such changes should be made can be decided readily on the basis of the titrations.

In addition to the solvent systems listed in Table 1, others were tested, tentatively in most cases. These were: aqueous

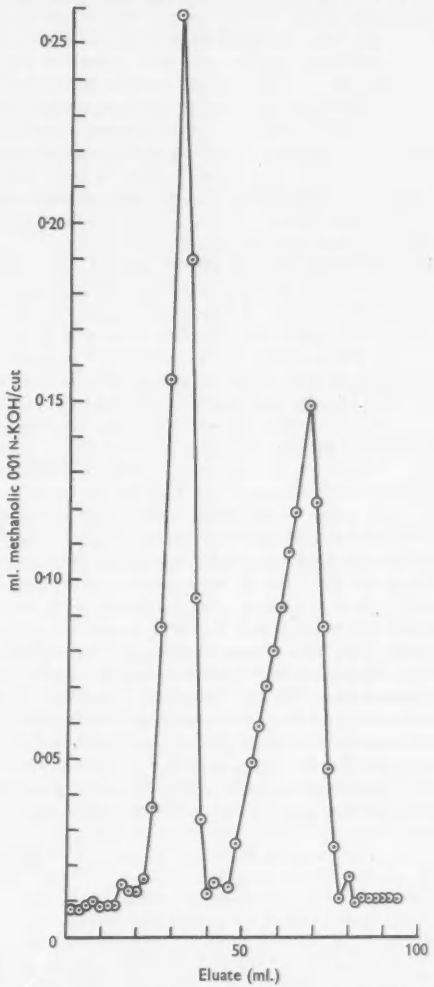

Fig. 7. The chromatographic separation of lauric and myristic acids. Moving phase, 70% (v/v) aqueous methanol saturated with *n*-octane; stationary phase, *n*-octane saturated with 70% (v/v) aqueous methanol. Ambient temperature 35°; recovery 98%.

methanol-toluene, aqueous methanol-$CHCl_3$, aqueous methanol-undecanol, aqueous ethylcellosolve-$CHCl_3$ and aqueous methanol-paraffin. The use of the last is exemplified in Fig. 2, but in view of the suitability of the aqueous methanol-octane and aqueous acetone-paraffin systems the use of the others was not pursued.

RESULTS AND DISCUSSION

The small amounts (about 2 mg.) of material, used on the columns precluded any identification of the acids in the eluate by normal physical means such as melting points. However, acids of authentic purity were employed in the work described here and single acids applied to the columns gave single peaks in the titration curve. There can thus be no doubt that the peaks observed represent the acids applied. The position of the peak is characteristic for an acid in a particular solvent system and the acids present in a mixture may be identified by comparing the position of their peaks with those given by known acids in the same solvent system.

Figs. 2–7 indicate that lauric, myristic, palmitic and stearic acids may be separated readily and the proportion of each in the mixture determined. Aqueous methanol-*cyclo*hexane or *n*-octane and aqueous acetone-medicinal paraffin have been used effectively as the solvent systems. No saturated acids with more than 18 carbon atoms and no acids with an odd number of carbon atoms have been available for testing, but brassidic and erucic acids behave well and no doubt the method could be applied to acids having more than 22 carbon atoms.

Table 1 shows some of the results obtained with various acids and solvent systems. The effect of lowering the percentage of water in the developing solvent on the rate of movement of any acid is evident from the data; the more water there is present the more slowly the acid travels down the column. The band formed by any given acid tends to spread more widely when the water content of the solvent is too high; the behaviour of palmitic acid on the 70% (v/v) aqueous methanol-octane column is an example of this (Fig. 2). It will be noted that on the 65% (v/v) aqueous acetone-paraffin column oleic, elaidic and palmitic acids all travel at similar rates. Earlier experiments with oleic, petroselenic, linoleic, linolenic and eleostearic acids made it clear that with constant chain length increasing the degree of unsaturation increases the rate of movement down the column. Acids with different degrees of unsaturation but the same chain length should be separable, as the behaviour of a commercial 'purified' oleic acid (Fig. 5) indicates. It is also interesting to note from this figure that oleic acid behaves in a similar way to the dibromostearic acid derived from it. Elaidic acid has always been found to travel down a column slightly more slowly than oleic acid, and the erucic-brassidic acid pair behaves similarly. This is in agreement with the general idea that the *trans* acids are more like the saturated acids than the unsaturated acids in their physical behaviour. The hydroxyacids are readily separated from both saturated and unsaturated acids by reversed-phase partition chromatography. Ricinoleic acid was readily purified in this way and the dihydroxystearic acids derived from oleic acid travel very quickly down the columns. Saturated and unsaturated acids could be readily separated by oxidizing the mixture before chromatography and the hydroxyacids themselves are being studied as a basis for analysing mixtures of unsaturated fatty acids.

SUMMARY

1. A new method of partition chromatography has been devised in which the less polar of the two phases is the stationary phase.

2. This reversed-phase method has been applied to the separation of the long-chain fatty acids.

3. Saturated acids having 12 to 18 carbon atoms have been separated, and mixtures of saturated, unsaturated and substituted acids have been analysed.

Our thanks are due to Prof. T. P. Hilditch, F.R.S., Dr W. T. J. Morgan, F.R.S, and Dr R. E. Bowman for gifts of fatty acids, to Prof. R. P. Linstead, F.R.S., for the dichlorodimethylsilane and to Miss J. Webb for technical assistance.

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Comparison of Electrophoretic Patterns of Human Sera obtained in Phosphate and in Diethylbarbiturate Buffer

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Diethylbarbiturate buffer of pH about 8·5 and ionic strength (I), 0·1 (Longsworth, 1942), is considered to be best suited for the electrophoretic analysis of human sera on account of the good resolution of the α_1-globulin from the albumin. With this buffer, however, which contains nitrogen, protein analyses cannot be made by the Kjeldahl method, the need for which may arise if fractions are to be studied further. Martin (1949) was the first to report analyses of pathological sera in which α_1-globulin was estimated in phosphate of pH 8 and ionic strength 0·2; but the electrophoresis may take up to 4 hr. Since it had been observed that the α_1-globulin separates from the albumin more readily in phosphate buffer at a pH around 8·8, the possibility of replacing diethylbarbiturate by this buffer was investigated.

EXPERIMENTAL

Serum or plasma was diluted to a protein concentration of about 1·4 g./100 ml. with a solution containing $Na_2HPO_4.2H_2O$, 8·71, or 17·42 g./l. ($I = 0·15$ or 0·3) or with diethylbarbiturate buffer as used by Longsworth (1942) of pH 8·6 and $I = 0·1$, and dialysed at 0–4° for 2 days. The dilutions of serum equilibrated against the phosphate solution had a pH of 8·85 as measured with a glass electrode. The Tiselius apparatus with a cylindrical lens schlieren optical system was used. The patterns for the analysis were obtained with a diagonal wire (Svensson, 1939) throughout. The photographs of the ascending patterns were taken immediately after the current had been stopped, but those of the descending patterns after the β-anomaly had subsided sufficiently to show the outline of the curve clearly and within the measurable range. An example of the application of Pedersen's (1940) method for dividing the areas is given in Fig. 1. Each enlarged pattern was traced and analysed only once. The errors are therefore such as would be encountered in routine analysis. Care was taken in placing the base line so that the two ends of base line and pattern at which there was no elevation coincided exactly.

The values obtained from the ascending and descending patterns agreed within the error range of the mean values reported in the literature (Table 1*a*) (Dole, 1944; Seibert, Seibert, Atno & Campbell, 1947; and others). In Table 2, therefore, the mean of these values only is given in each case. Although, for theoretical reasons, exact agreement between the ascending and descending percentage values should not be expected, the uncertainty in dividing the areas does not seem to permit the measurement of these deviations, for experiments at low protein concentration and high buffer ionic strength.

The wattage through the whole apparatus was kept near 6 or slightly under (Svensson, 1946). The potential gradients used were about 9·5, 6·8 and 4·9 V./cm. and the times of electrophoresis were about 1·5, 1·5–2·5 and 2·7 hr. for the diethylbarbiturate and the two phosphate buffers respectively.

Table 1. *Distribution of the differences between the percentage values for all components*

Difference ...	0·0–1·0	1·1–2·0	2·1–3·0	3·1–3·7	Total
Frequency (*a*)	41	32	9	6	88
(*b*)*	18	9	6	1	34

(*a*) Ascending and descending patterns, (*b*) patterns obtained in phosphate and diethylbarbiturate buffer.

* Albumin α_1-globulin taken together.

RESULTS

In Fig. 2 the patterns of a normal serum and one of a patient who had cirrhosis of the liver are shown. The analyses of these and four other sera are given in Table 2. There was no evidence that the results for (albumin + α_1-), α_2-, β- and γ-globulin, obtained with phosphate of one ionic strength were in better agreement than the other with the results obtained using diethylbarbiturate; or that the two analyses using phosphate differed from each other. The distribution of the differences between the percentage values for (albumin + α_1-), α_2-, β- and γ-globulin in phosphate, including one case of phosphate at pH 8 and I 0·1, and in diethylbarbiturate, for total protein concentrations of 1·5 g./100 ml. or below are given in Table 1*b*. Theoretically the greatest discrepancies should be expected in the values for α_1-globulin and albumin, since their relative difference in the mobility is the smallest. The percentages of α_1-globulin did not differ significantly, but in phosphate at ionic strength 0·15 the percentages of albumin were, with one exception, always higher than in diethylbarbiturate (average difference + 2·0). This was probably a consequence of 'boundary anomalies'. That boundary anomalies are noticeable in phosphate at $I = 0·15$ may be seen in the experiments with serum 5 (Table 2), which was run at three different concen-

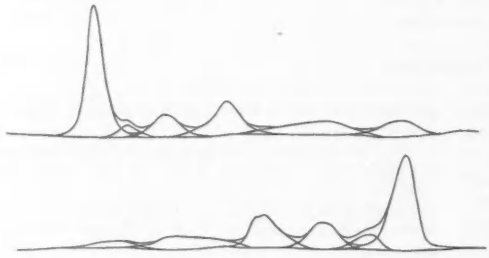

Fig. 1.

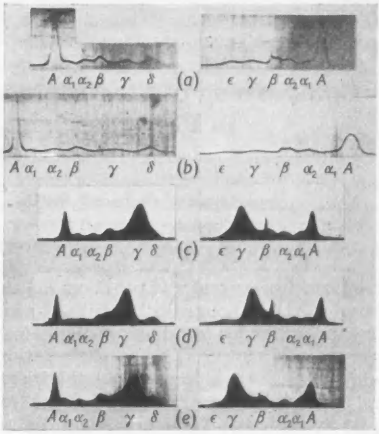

Fig. 2.

Fig. 1. Tracings of ascending and descending patterns of serum no. 6 at a protein concentration of 0·75 g./100 ml.

Fig. 2. Electrophoretic patterns of sera. *a* and *b*, pooled normal serum; no. 2 of Table 2; *c–e*, serum from patient with cirrhosis of liver; no. 4 of Table 2; (*a*) and (*c*) in diethylbarbiturate buffer, pH 8·6, *I*, 0·1; (*a*) after 99 min. at 9·5 V./cm.; (*c*) after 92 min. at 9·5 V./cm.; (*b*) and (*e*) in Na_2HPO_4, *I*, 0·15; (*b*) after 135 min. at 7 V./cm.; (*e*) after 105 min. at 6·8 V./cm.; (*d*) in Na_2HPO_4, *I*, 0·3, after 160 min. at 4·9 V./cm.

Table 2. *Apparent composition of serum or plasma in diethylbarbiturate and phosphate buffers*

(The figures for diethylbarbiturate are the mean percentages of the ascending and descending sides, those for phosphate are the differences in the percentage values from these means.)

Designation: serum proteins (g./100 ml.), sample diluted to protein concentration of g./100 ml.	Buffer	Albumin	Globulins α_1-	Globulins α_2-	Globulins β- (+ϕ)	Globulins γ-	Albumin + α_1-globulin	$\alpha_1+\alpha_2+\beta$
1. Normal plasma, 7·2; 1·4	Diethylbarbiturate Na_2HPO_4	57·3	5·2	9·5	19·6	8·4	62·5	—
	I=0·3	−3·0	+1·5	−0·9	+0·1	+2·3	−1·5	—
	I=0·15	−0·7*	+2·1*	−1·1	0·0	+0·5	+0·6	—
2. Normal serum pool, 7·3; 1·4	Diethylbarbiturate Na_2HPO_4	51·7	7·9	11·1	14·3	15·0	59·6	—
	I=0·15	+2·4	−2·2	+0·5	−0·5	−0·2	+0·2	—
3. Multiple myeloma serum, 7·6; 1·4	Diethylbarbiturate Na_2HPO_4	42·8	3·7	9·7	10·9	32·9	—	24·3
	I=0·3	−0·2	(+0·5	−2·7	+4·7)	−2·3	—	+2·5 bad separation of α_1-, α_2- and β-globulin
	I=0·15	+2·1	+0·5	−1·3	−0·3	−1·1	+2·6	−1·1
4. Cirrhosis of liver serum, 11·3; 1·4	Diethylbarbiturate Na_2HPO_4	18·8	3·8	5·5	71·9		22·6	—
	I=0·3	−0·4	+1·1	+0·4	−1·9		+1·5	Tilt 15°
		+0·4	+1·4	+1·1	−2·9		+1·8	Tilt 25°
	I=0·15	+1·7	+0·4	+0·1	−2·2		+2·1	—
5. Pregnancy serum, 7·7	1·5 Diethylbarbiturate	45·2	7·9	15·7	16·7	14·5	53·1	—
	0·75 Na_2HPO_4	+1·6	−1·9	−2·8	+2·0	+1·1	−0·3	—
	1·5 I=0·15	+2·8	−1·2	−3·5	+1·7	+0·2	+1·6	—
	2·25	+4·6	−1·9	−4·2	+1·2	−0·3	+2·7	—
6. Normal serum, 7·3; 1·2	Diethylbarbiturate	53·1	5·4	8·9	13·7	18·9	58·5	—
	Phosphate pH 8, I=0·1	—	—	−0·4	+0·1	0·0	+0·3	—

* From ascending pattern only.

trations of protein. The rate of increase in the apparent proportion of albumin with protein concentration was 2·0 %/g./100 ml. Perlmann & Kaufman (1945) have measured the same relationship in diethylbarbiturate buffer. From their Fig. 3 the rate of increase worked out at about 2·1. Although comparison is not strictly possible, since the ratio of albumin to α_1-globulin was larger than in serum 5, it may be said that the variation of the apparent proportions with total protein concentration in phosphate at ionic strength 0·15 is probably not greater than that in diethylbarbiturate at ionic strength 0·1. The larger the ratio albumin/α_1-globulin, the smaller should be the variation in the apparent proportion of the albumin.

The analyses of serum 6 confirmed the agreement observed earlier (Hoch, 1948) between the results obtained with diethylbarbiturate of pH 8·6 and $I = 0{\cdot}1$ and phosphate buffer pH 8 and $I = 0{\cdot}1$.

DISCUSSION

Diethylbarbiturate and phosphate buffers were compared with each other on the assumption that the fractions separated are the same in both, though this need not be the case. In experiments with sera 2 and 5 (Table 2) it might be suggested that in phosphate part of the α_1-globulin has been included in the albumin, or conversely with serum 1, that this has happened in diethylbarbiturate. It does not seem easy to test this because of the uncertainty in extrapolating the curve of the albumin, which was assumed to be symmetrical.

With one serum the separation of α_1-globulin from albumin was better in phosphate at $I = 0{\cdot}3$ than at 0·15, while with another α_1-, α_2- and β-globulin separated better at $I = 0{\cdot}15$. The use of phosphate at $I = 0{\cdot}3$ is not practicable for routine examinations because the time of electrophoresis is unduly long, and because the spreading of the globulin boundaries causes too much overlapping. The discrepancies between diethylbarbiturate and phosphate, at a total protein concentration of 1·4 g./100 ml., were within the experimental error of dividing the areas, except for albumin. The magnitude of these discrepancies is clinically of little significance. One may suggest, therefore, that for routine clinical work disodium phosphate can replace diethylbarbiturate if the concentration of total protein is between 1·0 and 1·4 g./100 ml. While the resolution of the components in diethylbarbiturate buffer is superior without doubt, phosphate may be preferred when protein estimations have to be made by the Kjeldahl method. The greater cost of diethylbarbiturate may also be taken into consideration.

Phosphate at pH 8·8 has very little buffering capacity. This, however, is of no serious concern to the experiments described here, since the protein itself exerts sufficient buffering action in all sections of the U-tube except where the concentration is small, such as in the region of the γ-globulin of normal sera on the descending side. The effect of a higher pH would be to increase the migration velocity of the γ-globulin in this region, resulting in a higher apparent relative proportion of the γ-globulin. The error introduced by this is opposite to that due to the boundary anomalies. No significant differences in the percentage values for the γ-globulin were found.

In some of the runs in phosphate slight convexional disturbances were noticed ahead of the descending albumin boundary, such as can be seen in Fig. 2*d*. These may have been caused by the separation of a faster component in conjunction with the heat generated by the electric current.

SUMMARY

1. The α_1-globulin of human serum can be resolved in phosphate at a pH around 8·8.

2. The differences in the apparent relative proportions of the components of serum in this buffer and in diethylbarbiturate buffer of pH 8·6 were less than 3 % of the total area in 33, and less than 2·1 % in 27 out of 34 cases.

3. These differences are not of clinical significance and phosphate at pH 8·8 and $I = 0{\cdot}15$ may therefore be used in place of diethylbarbiturate buffer at pH 8·6 and $I = 0{\cdot}1$.

It is a pleasure to thank Prof. J. R. Marrack for his constant help and encouragement. Thanks are due to Mr R. G. S. Johns for making the pH measurements.

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The Influence of L-Ascorbic Acid on the Disappearance of the Phenolic Group of L-Tyrosine in the Presence of Guinea Pig-Liver Suspensions

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It is now well established that when high doses of L-tyrosine are consumed by guinea pigs or men, the phenolic group in the amino-acid is less readily metabolized if they subsist on a diet low in L-ascorbic acid than if a diet containing sufficient of this compound to saturate the tissues is offered (cf. Sealock & Silberstein, 1940; Levine, Marples & Gordon, 1941*a*, *b*). Evidence has, however, been produced (Painter & Zilva, 1947, 1948) which justifies the conclusion that this interaction between L-ascorbic acid and L-tyrosine is a physiological response to an unusual situation and has no important bearing on normal nutrition. The part played by L-ascorbic acid is, nevertheless, of biochemical interest, and the *in vitro* experiments described in this communication throw some light on the problem.

Pioneer work on the degradation of the molecule of L-tyrosine in the presence of animal tissue has been performed by Bernheim & Bernheim (1934), Krebs (1935) and Felix, Zorn & Dirr-Kaltenbach (1937). In these investigations the oxygen absorption and the evolution of ammonia techniques were employed. In the present work, as we were entirely concerned with the metabolism of the phenolic group of the molecule, we limited ourselves to the use of the Millon reaction only.

Darby, DeMeio, Bernheim & Bernheim (1945) were unable to establish any difference in the rate of disappearance of the phenolic group of L-tyrosine when incubated with liver tissue from normal and from scorbutic guinea pigs for 4 hr. As will be seen from the following experiments, under our experimental conditions we had no difficulty in demonstrating that such a difference does exist, and we were in consequence in a position to investigate the problem in some detail.

EXPERIMENTAL

Technique

Experimental animals and diet. All animals employed in this investigation were healthy young growing guinea pigs weighing approx. 300 g. The vitamin C-saturated animals were maintained on a diet of oats, bran and cabbage *ad lib.* and are referred to in text as normal or saturated animals. All the deficient animals received a scorbutic diet (Penney & Zilva, 1946) for 21 days.

Preparation of liver suspensions. The guinea pigs were killed by stunning and bleeding. The liver was removed and ground thoroughly in a mortar with a small quantity of sand according to the procedure of Bernheim & Bernheim (1934). Usually the livers of two animals were pooled. After adding the requisite quantity of 0·05 M-Na_2HPO_4-KH_2PO_4 buffer (pH 7·8), the mixture was further ground and then squeezed through fine muslin. The resulting suspension, which was readily transferable by means of a pipette, was used as soon as possible after preparation. Felix *et al.* (1937), Felix & Zorn (1941) and Sealock & Goodland (1949) suggest that in order to stabilize the system more completely the concentration of the buffer should be 0·2 M. Experiments in which parts of the same livers were ground with 0·05 and 0·2 M-buffers yielded in our hands similar results, and we therefore employed the former concentration.

Determination of hydroxyphenyl compounds. This was carried out by means of the Folin and Ciocalteu method (Painter & Zilva, 1947), except that a photoelectric instead of a visual colorimeter was employed in this investigation. L-Ascorbic acid did not appreciably influence the colour production unless the quantities added exceeded 10 mg./flask when the colour tended to be low.

Method of incubation. Liver suspension (2 ml.) was pipetted into 25 ml. conical flasks which also contained 1 ml. of buffer or 1 ml. of a buffered solution of L-ascorbic acid according to requirements. A buffered solution (1 ml.) containing 2 mg. of L-tyrosine or an amount of glycyl-L-tyrosine which gave a colour value equivalent to that of the tyrosine was then added to each flask, except the control flasks. Duplicate flasks were used in each experiment. The unstoppered flasks were shaken 80 times/min. with an amplitude of 12 cm. in a bath at 37·5°. At the requisite time the reaction was stopped by the addition of 4 ml. of 5% (w/v) HPO_3, after which 1 ml. of the L-tyrosine or dipeptide solution was added to the control flasks. After centrifuging the contents of the flasks, the hydroxyphenyl compounds were determined using 0·5 ml. for the test.

Preliminary experiments have shown that the optimum activity lies between pH 7·3 and 7·5, an observation which is in agreement with that made by Bernheim & Bernheim (1934) and by Sealock & Goodland (1949). We therefore maintained our reaction media at pH 7·3–7·5.

As it was found that the variation of the individual activity values about the mean was quite large even when the 0·2 M-buffer was used (cf. Sealock & Goodland, 1949), we employed experimental groups which contained six or more animals.

L-Tyrosine and glycyl-L-tyrosine. See Painter & Zilva (1947).

L-Ascorbic acid. Kindly supplied by Roche Products Ltd. Solutions of the acid were prepared immediately before use.

D-Glucoascorbic acid. Sample kindly presented by Prof. E. L. Hirst. Brownish amorphous powder, purity approx. 85%.

RESULTS

The disappearance of the phenolic group of L-tyrosine and glycyl-L-tyrosine in the presence of suspensions of normal and of scorbutic guinea pig livers

We found that the diminution of the phenolic reaction during the first hour of incubation could not be accurately differentiated between the two groups by our technique, owing no doubt to the small quantity of L-tyrosine degraded during this period and we therefore omit these figures. Fig. 1 gives the

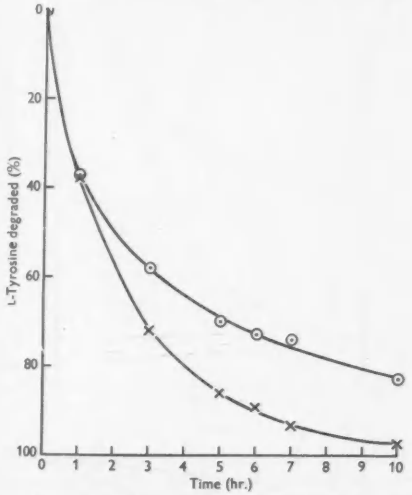

Fig. 1. The rate of disappearance of the phenolic group of L-tyrosine in the presence of suspensions of guinea pig livers; ×——×, livers from normal animals; each point represents the mean value of thirteen pairs; ⊙——⊙, livers from scorbutic animals; each point represents the mean value of ten pairs.

results obtained after periods varying from 1 to 10 hr. The percentage of L-tyrosine which had disappeared after 3 hr., when normal livers were used, was 72 (S.D. 10·8), and in the case of scorbutic livers the percentage was 58 (S.D. 9·5). Combined results of experiments in which the liver suspensions from a larger number of animals, namely forty-six pairs of normal and forty-eight pairs of scorbutic guinea pigs, was used showed that after 3 hr. incubation figures of the same order as above were obtained (68, S.D. 10·5 and 54, S.D. 11·8, respectively). By using Student's t test, the differences were shown to be statistically significant ($P < 0{\cdot}01$ in the former set of experiments and $< 0{\cdot}001$ in the latter). Similarly, the differences observed between the two groups after longer periods of incubation (Fig. 1) were also found to be significant. Thus it is perfectly plain that the phenolic group disappeared at an appreciably higher rate in the presence of liver suspensions originating from the normal saturated animals than from scorbutic animals, the tissues of which were almost completely devoid of L-ascorbic acid. In both cases the logarithmic shape of the curves was similar to that obtained by Bernheim & Bernheim (1934) and by Felix *et al.* (1937), who studied the absorption of oxygen by liver suspensions from normal animals only. It may be noted that even with such suspensions the reaction proceeds comparatively slowly, which is also in consonance with the results obtained by the above workers. Lan & Sealock (1944) have observed that while liver slices from normal guinea pigs absorbed extra oxygen in the presence of L-tyrosine the same tissue from scorbutic animals did not. This apparent deviation from our results is in our opinion due to the difference in technique employed in the respective investigations. This view is strengthened by the observation made later by Sealock, Goodland & White (1947) that the oxygen uptake by liver suspensions from scorbutic guinea pigs is influenced by the ratio of liver tissue to the substrate in the reacting medium more than when liver suspensions from normal animals are used. Further, it may also be pointed out that our tests did not measure the total oxidation of the L-tyrosine molecule, but indicated only the disappearance of the phenolic group.

We also found that when glycyl-L-tyrosine was used as the substrate the phenolic group, as in the case of L-tyrosine, disappeared after 3 hr. to a greater extent when suspensions of livers from normal animals were used. The metabolism of this substrate after longer periods was not investigated.

The disappearance of the phenolic group of L-tyrosine in the presence of suspensions of livers from scorbutic guinea pigs injected with L-ascorbic acid

This experiment was instituted in order to ascertain whether the higher rate of the disappearance of the phenolic group in the presence of suspensions of livers from normal animals was due to their higher content of L-ascorbic acid *per se*. For this purpose a number of guinea pigs were placed on a scorbutic diet for 21 days. They were injected intraperitoneally with 25 mg. of partially neutralized L-ascorbic acid 18 hr. before being killed. After this treatment such guinea pigs usually become saturated, whilst the quantity of L-ascorbic acid circulating in the system is not unduly high (Penney & Zilva, 1946). On the other hand, the macroscopic post-mortem picture in

such instances does not visibly differ from that observed in animals kept for 21 days on a scorbutic diet which were not previously treated with L-ascorbic acid. This post-mortem picture was observed in all our animals. Table 1 shows the L-ascorbic acid content of the liver and the percentage of L-tyrosine metabolized in 3 hr. For the determination of L-ascorbic acid a portion of the liver suspension was treated with trichloroacetic acid, centrifuged and analysed by the modified Roe and Kuether method (cf. Penney & Zilva, 1945). It will be seen from the figures that the L-tyrosine was metabolized to the same extent as that observed in the case of normal guinea pigs (mean of injected animals was 68 (S.D. 12·6); the mean of the normal animals was 68 (S.D. 10·5). Furthermore, there is a significant difference between the mean value obtained in this experiment and that found previously when livers from scorbutic animals were used (i.e. 54, S.D. 11·8, $P < 0·01$). The result of this experiment therefore suggests that the presence of L-ascorbic acid in the liver is responsible directly for the acceleration in the rate of the metabolism of L-tyrosine. Subsequent experiments will offer further support to this view.

Table 1. *The disappearance of the phenolic group of* L-*tyrosine after incubation for* 3 *hr. in the presence of suspensions of livers from scorbutic guinea pigs injected with* 25 *mg. of* L-*ascorbic acid* 18 *hr. before death*

	Individual values								Mean	S.D.
L-Ascorbic acid content of liver (mg./100 g.)	12·9	12·8	9·1	18·8	11·3	12·7	16·7	15·1	—	—
Percentage of L-tyrosine disappeared	70	93	66	50	76	69	73	60	68	12·6

The influence of graded quantities of L-*ascorbic acid on the disappearance of the phenolic group of* L-*tyrosine and glycyl-*L-*tyrosine in the presence of suspensions of livers from normal and scorbutic guinea pigs*

As the presence of L-ascorbic acid in the guinea pig-liver tissue is capable of accelerating the rate of metabolism of L-tyrosine it was of interest to establish to what extent the acceleration of the reaction was influenced by the quantity of L-ascorbic acid present. The influence of the addition of quantities ranging from 0 to 10 mg. to the reaction mixtures was therefore investigated after 3 hr. incubation. Control flasks containing L-tyrosine and L-ascorbic acid showed that the substrate remains unaltered in the absence of liver suspensions. The results are given in Fig. 2. As was to be expected, the addition of L-ascorbic acid to the suspensions of livers from scorbutic animals accelerated the reaction. It was found, for example, that when 1 mg. was added the percentage of L-tyrosine that had disappeared was raised from 50 (S.D. 12·2) to 66 (S.D. 11·7) the difference between the two values being significant ($P < 0·05$). This was also the case when livers of normal animals were used, except that here no statistical significance was observed until 2·5 mg. of L-ascorbic acid were added, the percentage being raised from 65 (S.D. 9·7) to 78 (S.D. 11·7) ($P < 0·05$). Nevertheless, beyond a certain limit of concentration, namely when 2·5 mg. were added, the L-ascorbic acid not only failed to exercise any further accelerating action, but seemed to inhibit somewhat the rate at which the phenolic group of the substrate disappeared. It is also to be noticed that as much as 1 mg. of L-ascorbic acid had to be added to the liver suspensions from the scorbutic animals in order to attain the same degree of L-tyrosine degradation after 3 hr. incubation as that observed with liver

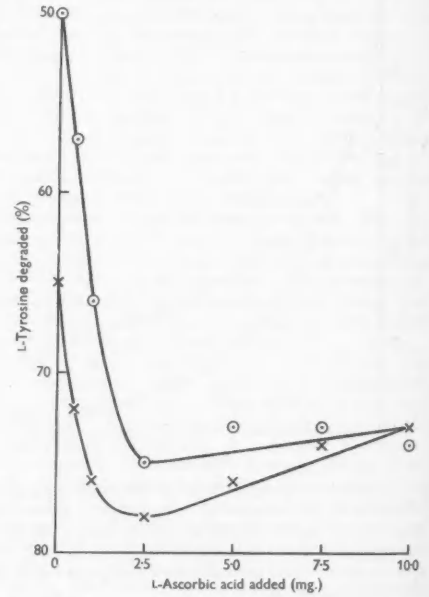

Fig. 2. The influence of graded amounts of L-ascorbic acid on the disappearance of the phenolic group of L-tyrosine in the presence of suspensions of guinea pig livers after 3 hr. incubation; ×——×, livers from normal animals; each point represents the mean value of seven pairs; ⊙——⊙, livers from scorbutic animals; each point represents the mean value of seven pairs.

suspensions from normal animals without the addition of L-ascorbic acid. This latter reaction mixture contained only about 0·2 mg. of L-ascorbic acid all of which originated from the liver tissue.

The above experiments were repeated with glycyl-L-tyrosine as the substrate, and results very similar to those obtained with L-tyrosine as substrate were observed except that the initial values were lower.

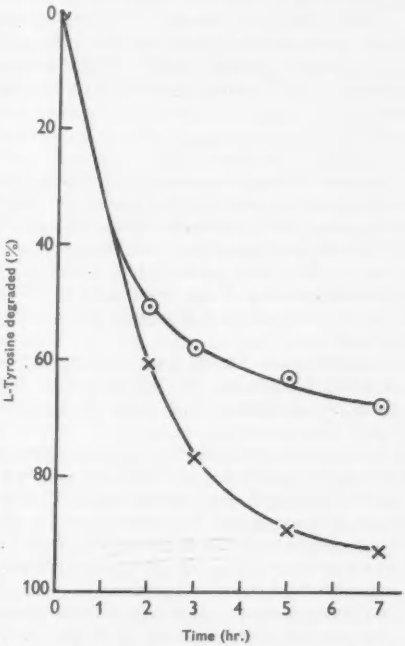

Fig. 3. The effect of 2·5 mg. of L-ascorbic acid on the rate of disappearance of the phenolic group of L-tyrosine in the presence of suspensions of livers from scorbutic guinea pigs; ×——×, no L-ascorbic acid added; each point represents the mean value of seven pairs; ⊙——⊙, L-ascorbic acid (2·5 mg.) added; each point represents the mean value of seven pairs.

Fig. 3 shows the rate at which the phenolic group of L-tyrosine disappeared after the addition of the optimum amount of 2·5 mg. of L-ascorbic acid to liver suspensions from scorbutic guinea pigs. It will be seen that the curve obtained is almost identical in shape with that found when liver suspensions from normal animals were used (Fig. 1). In this connexion it is of interest to mention that Lan & Sealock (1944), using slices of livers from scorbutic guinea pigs and L-tyrosine as substrate, found an increase in the oxygen uptake by adding 0·3 mg. of L-ascorbic acid to the reaction mixture.

The influence of D-*glucoascorbic acid on the disappearance of the phenolic group of* L-*tyrosine in the presence of suspensions of livers from normal and scorbutic guinea pigs*

As it was probable that the influence of L-ascorbic acid in accelerating the metabolism of L-tyrosine in this reaction was due to its oxidation-reduction potential, an experiment was instituted in which L-ascorbic acid was replaced by D-glucoascorbic acid. Six normal and four scorbutic animals were used for this purpose. It will be seen from Table 2 that when

Table 2. *The effect of* D-*glucoascorbic acid* in vitro *on the disappearance of the phenolic group of* L-*tyrosine after* 3 *hr. incubation with guinea pig-liver suspensions*

(Results expressed as percentages of L-tyrosine originally present.)

Normal animals		Scorbutic animals	
No addition	D-Glucoascorbic acid equivalent to 2·5 mg. of L-ascorbic acid added	No addition	D-Glucoascorbic acid equivalent to 5·0 mg. of L-ascorbic acid added
62	80	42	59
79	87	52	61
50	74	67	71
79	90	59	94
73	80	—	—
62	75	—	—
Mean 67	81	—	—
S.D. 11·5	6·4	—	—

a quantity of D-glucoascorbic acid, equivalent to 2·5 mg. of L-ascorbic acid, was added to the reaction mixtures in which suspensions of livers from normal guinea pigs were used the percentage of L-tyrosine which was metabolized in 3 hr. was significantly raised from 67 (S.D. 11·5) to 81 (S.D. 6·4) ($P < 0·05$). Unfortunately, in the case of the scorbutic guinea pigs, we were unable to use a number of animals which would yield figures for a satisfactory statistical analysis. The results, however, indicate that in this case the disappearance of the substrate was also accelerated by the presence of D-glucoascorbic acid.

DISCUSSION

It emerges from this investigation that the phenolic group in L-tyrosine, when present in a concentration higher than that which is likely to occur in any of the tissues *in vivo* under normal conditions, is degraded slowly *in vitro* in the presence of guinea pig-liver suspensions. The rate of degradation is accelerated by the presence of L-ascorbic acid, whether the acid originates from the liver or whether it is added to the reaction mixture in the form of the synthetic compound. This is in consonance with the observations

made *in vivo* on guinea pigs which received high doses of L-tyrosine. It would also appear that the higher rate of degradation observed, when livers from saturated guinea pigs were used than when those from scorbutic guinea pigs were employed, is due to the higher quantities of L-ascorbic acid present in the former and that the vitamin acts directly *per se*. Lan & Sealock (1944) have shown that the livers from scorbutic guinea pigs which had been previously dosed with 20 mg. of L-ascorbic acid daily for 6 days reacted like livers from normal guinea pigs. The animals after such treatment must have been, as is usual, free from the reversible lesions which make up the syndrome of scurvy. In our experiments, on the other hand, owing to the short time that elapsed between the injection of the L-ascorbic acid and the death of the animals, the post-mortem picture which we observed was similar to that obtained with scorbutic animals not previously treated with L-ascorbic acid; yet the action of the liver suspension was the same as that from livers of normal animals. It is therefore reasonable to assume that this was due to the saturation of the tissues with the vitamin.

The results obtained in the experiment in which D-glucoascorbic acid replaced L-ascorbic acid show that the accelerating action of the compounds is in all probability due to their oxidation-reduction potential. D-Glucoascorbic acid does not possess any antiscorbutic activity, because unlike its active enantiomorph it is not retained by the tissues but is entirely excreted by the kidneys (Zilva, 1935). It is therefore interesting to find that when brought into contact with the substrate *in vitro* it functions like the antiscorbutically active L-ascorbic acid. This offers an explanation why D-araboascorbic acid, which is partially retained by the tissues of the guinea pig (cf. Zilva, 1935), when administered to these animals with high doses of L-tyrosine, was found by Sealock & Silberstein (1940) to be not as efficient as L-ascorbic acid in accelerating the disappearance of the phenolic group of L-tyrosine.

That L-ascorbic acid acts as a catalyst in this reaction is evident from the fact that the quantities of L-ascorbic acid which disappeared during the experiment in the flasks in which the reaction took place was found by us to be the same as that which disappeared in the control flasks from which L-tyrosine was absent, in other words, none of the acid was used up in the actual reaction.

The evidence produced in these experiments does not offer sufficient information to formulate a complete hypothesis of the part played by L-ascorbic acid in the mechanism of the metabolism of L-tyrosine by the enzyme system of the liver, especially as the degradation of only one part of the molecule was investigated by us. The results invite further investigation.

SUMMARY

1. The rate of disappearance of the phenolic group of L-tyrosine in the presence of suspensions of livers from normal or from scorbutic guinea pigs was investigated over periods up to 10 hr. The reaction was found to be slow and proceeded at a higher rate when suspensions from livers of normal animals were used. Glycyl-L-tyrosine could replace the L-tyrosine as substrate.

2. Suspensions of livers from scorbutic guinea pigs injected with 25 mg. of L-ascorbic acid 18 hr. before death functioned like those from normal animals.

3. The accelerating effect of graded quantities (0·5–10 mg.) of L-ascorbic acid was investigated in the presence of liver suspensions from normal and scorbutic animals using L-tyrosine and glycyl-L-tyrosine as substrates. The addition of 2·5 mg. of the acid was found to produce the optimum acceleration. No L-ascorbic acid was used up in the reaction.

4. D-Glucoascorbic acid, which is antiscorbutically inactive, accelerated the above degradation *in vitro* to the same extent as equivalent amounts of L-ascorbic acid.

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Synthesis of Cholesterol and Fatty Acids in Foetuses and in Mammary Glands of Pregnant Rabbits

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The aim of this investigation was to study the sources of foetal fatty acids and cholesterol, i.e. to decide whether these substances are derived from the mother by placental transmission or are synthesized within the foetus from suitable precursors. Further, it was hoped that by studying foetal organs separately information might be obtained about their role in the fat metabolism of the foetus.

It was shown in previous investigations (Popják, 1947; Popják & Beeckmans, 1950) that phospholipins in the rabbit foetus are formed by foetal synthesis and that although the placenta of foetal origin absorbs much phospholipin from the maternal blood it does not transmit unhydrolysed phospholipin molecules to the body of the foetus. Certain observations on the fat content of foetuses from cholesterol-fed mothers could be explained on the assumption that fatty acids and cholesterol are also chiefly synthesized within the foetus from smaller molecules, and it was pointed out that this problem could be solved with the aid of isotopes (Popják, 1946). While this investigation was in progress Goldwater & Stetten (1947) provided evidence that fatty acids and cholesterol are mainly synthesized in the rat foetus, as judged by the rapid incorporation of deuterium (D) into these substances from the body water. In addition they showed that, when D-labelled fatty acids were fed to the mothers for 2 days (between the eighteenth and the twentieth days of gestation), not more than 1·5% of the foetal fatty acids were derived from the maternal dietary fat. When deuteriocholesterol was fed for 7 days (between the thirteenth and twentieth days of gestation) to pregnant rats, approximately 10·7% of the foetal cholesterol was derived from the dietary cholesterol.

The results of this investigation, carried out on rabbits, generally confirm the findings of Goldwater & Stetten (1947) on rats and provide additional proof by showing the utilization of acetate-carbon for fatty acid and cholesterol synthesis in the foetus. The said authors, however, studied only the unfractionated total fatty acids and cholesterol in the foetus as a whole and their investigation gives no information about the role of the placenta, liver or extrahepatic tissues of the foetus in lipid synthesis. The question whether foetal lipids are synthesized within the body of the foetus or in the placenta and transported from there was left entirely open. In the present investigation particular attention was paid to these points. Further, in a quantitative analysis of the results a comparison was made between the rate of accumulation and rate of synthesis of the fatty substances in the growing foetus in order to assess whether growth is associated primarily with increased synthetic activity or decreased degradation, or both.

The classical experiments of the Schoenheimer school (Schoenheimer, 1941) have shown that the rate of incorporation of D into fatty acids and cholesterol in animals whose body water has been enriched with D_2O for varying lengths of time, gives a good measure of the rates of synthesis and degradation of these compounds. The first part of the present investigation is concerned with the rate of synthesis of D into foetal cholesterol and fatty acids. In later sections the utilization of acetate in the foetus for the synthesis of these compounds will be demonstrated, and observations on fat formation in the mammary gland of pregnant, non-lactating rabbits will also be reported.

To obtain evidence concerning the question whether the foetal lipids are derived from the preformed maternal compounds by placental transmission or by synthesis within the foetus, a comparison between the isotope content of the maternal plasma lipids and foetal lipids should be made. If the latter were to exceed the former under the experimental conditions to be outlined, the result would indicate foetal synthesis. Unfortunately, material sufficient for D analysis by the falling-drop method used in this investigation, cannot be obtained from the plasma of one rabbit, particularly as rabbits develop a lipopenia during the second half of pregnancy (Baumann & Holly, 1925–6; Popják, 1946).

The D content of fatty acids and cholesterol of the maternal liver was therefore compared with that of the foetal tissues on the assumption that the plasma lipids are derived from the liver or are in equilibrium with liver lipids. If this assumption is correct, then under our experimental conditions the D content of the maternal plasma lipids cannot exceed that of the liver lipids. It is known that plasma phospholipins in the adult animal are synthesized in the liver (Fishler, Entenman, Montgomery & Chaikoff, 1943;

Entenman, Chaikoff & Zilversmit, 1946). Results obtained in animals injected with $CH_3{}^{14}COONa$ indicate that the source of plasma cholesterol is also probably the liver. The available evidence, therefore, seems to justify the assumption on which the interpretations of the results of this investigation are based.

METHODS

Rabbits (2·5–3·0 kg.) were inseminated artificially, and hence the period of gestation was known within narrow limits. They were first injected with gonadotrophic hormone (50 i.u.) and were inseminated (0·2 ml. of semen) within 1 hr. Under such conditions conception should take place during the 12 hr. period after the injection of gonadotrophic hormone.

On a chosen day during pregnancy (between the 16th and 28th day) the animals were given intravenously an amount of pure D_2O (made isotonic with NaCl) which brought the concentration of D_2O in the body fluids to 1·5%; the drinking water was then replaced by 2% D_2O in tap water. The concentration of D_2O in the body fluids was thus maintained constant at a level of about 1·5% for 1–12 days. All experiments were terminated on the 28th day of pregnancy. If, therefore, the animals were kept on D_2O for n days, the beginning of the deuterium regimen was on the $(28-n)$th day. This particular mode of administration of D_2O to a growing system, like the foetus, allows a comparison between the rate of synthesis and the rate of accumulation of a particular substance, as will be shown in the discussion. The alternative choice of giving D_2O for n days starting, say, on the 16th day and ending the experiment on the $(16+n)$th day is unsuitable for such purpose.

On the 28th day of pregnancy the animals were anaesthetized with nembutal and ether and the foetuses delivered one by one as described previously (Popják, 1946). Foetal blood was obtained from the umbilical vein and maternal from the aorta; both were heparinized.

Preparation of tissues. The excised livers of foetuses from each litter were pooled; carcasses were also pooled. The placentae were freed from the membranes and the foetal and maternal parts separated. Only the foetal part, i.e. that derived from the ovum, was investigated systematically. The lipids of the maternal portion of the placenta were examined only in two experiments, in which the animals were injected with $CH_3{}^{14}COONa$. The mammary glands of the mothers were dissected out and freed as far as possible of connective and adipose tissue.

All tissues were minced and ground to a fine pulp; the foetal carcasses were first minced and then reduced to a pulp in a Waring blender.

Samples were taken from the maternal liver and blood, the placenta, foetal liver and carcass for the distillation of body water in a high vacuum system. The D content of these water samples was determined and found to be identical within experimental errors.

Extraction and fractionation of lipids. The pulped tissues were extracted four times by boiling with ethanol-ether (3:1, v/v) for 5 min. each. The total volume of solvent used was at least 30 times the weight of the tissue. Negligible amounts of fatty material remain in the tissues after such extraction. The filtered and combined extracts were evaporated to dryness under reduced pressure at 30–40° in an atmosphere of N_2. The residues were extracted with boiling light petroleum (b.p. 40–60°) and the extract freed from suspended matter by centrifuging. The clear petroleum extracts were concentrated to a suitable small volume and the phospholipins precipitated by the addition of acetone and a saturated ethanolic solution of $MgCl_2$. The phospholipins were allowed to settle overnight in the refrigerator. After centrifuging, the acetone supernatant was poured off. The phospholipins were taken up in light petroleum and re-precipitated.

The phospholipins were dissolved in 10–20 ml. of moist ether and saponified by the addition of an equal volume of 80% ethanol and 40% (w/v) aqueous KOH (5 ml./g. of lipid) at 70–80° for 24 hr. As the saponification progressed, more and more water was added and at the end all the organic solvent was evaporated off. The soaps were transferred with hot water to separatory funnels, made acid with H_2SO_4 and the fatty acids extracted with light petroleum.

The acetone supernatants, obtained from the phospholipin precipitations and containing glycerides, together with free and combined cholesterol, were evaporated to dryness by distillation and the residues saponified with ethanolic KOH at 80° for 6 hr.; 10 ml. of ethanol and 1 ml. of 40% (w/v) aqueous KOH were used/g. of glyceride fat. When the saponification was finished the material was transferred with hot 50% (w/v) ethanol to separatory funnels and the unsaponifiable matter removed by extraction with light petroleum. Five petroleum extractions were carried out, after which the organic solvents were evaporated off from the soaps on a water bath. The free fatty acids were obtained as described for phospholipins. The light petroleum was evaporated off and the fatty acids dried over $CaCl_2$ and paraffin. These fatty acids will be designated as glyceride fatty acids, although a very small portion of them were derived from cholesteryl esters also. The glyceride fatty acids obtained from maternal and foetal liver and foetal carcass were also separated into saturated and unsaturated acids by the lead-salt method (Twitchell, 1921).

The extract of unsaponifiable matter was concentrated nearly to dryness, and 5–10 ml. of acetone were added. Cholesterol was precipitated by the addition of 0·5% (w/v) digitonin solution in 80% ethanol. The digitonide was washed twice with acetone-ether (2:1, v/v) and twice with ether and then dried, first at 70° and then *in vacuo* over P_2O_5.

Determination of deuterium in fatty acids and cholesterol. The organic substances were burnt and the water of combustion collected and purified as described by Keston, Rittenberg & Schoenheimer (1937–8). The deuterium contents in the water of combustion and in the water obtained from the distillation of tissue samples were determined by the falling-drop method (Barbour & Hamilton, 1926). *o*-Fluorotoluene was used as the medium through which the drops were allowed to fall. The temperature of the bath was maintained within $\pm 1{\cdot}5\times10^{-3}$°. A brief description of the constant-temperature bath and micropipette used for delivery of drops will be given in Appendix 1.

In the calculation of D content of cholesterol from the deuterium content of cholesterol-digitonide it was assumed that in 1 molecule of cholesterol-digitonide 46 H atoms of the cholesterol were diluted with 92 H atoms derived from digitonin, an assumption verified by experiment.

The expression of 'atom-% excess' for the D content of cholesterol and fatty acids will be used in this paper to denote the D content (atom-% excess) of these expressed as the percentage of the D content (atom-% excess) of the body water.

RESULTS

In all our experiments, whether of 1- or 12-day duration, the foetal cholesterol and fatty acids contained a higher concentration of D than the corresponding substances in the maternal liver (Tables 1–4). The results, therefore, generally indicate that fatty acids and cholesterol are synthesized within the foetus, thus confirming the findings of Goldwater & Stetten (1947). There are, however, significant differences in the isotope contents of these substances in the various foetal tissues. The further analysis of the results will be directed towards assessing the role of the foetal liver, the extrahepatic tissues and of the placenta in foetal-fat metabolism.

Synthesis of deuterium into foetal cholesterol. From Table 1 it can be seen that the most rapid incorporation of D from body water into cholesterol occurs in the foetal liver, the next in order being the foetal carcass. In 11 and 12 days the D concentration in the cholesterol of both foetal liver and foetal carcass seems to have reached a maximum concentration of about 63 atom-% excess. Rittenberg & Schoenheimer (1937) have shown that the maximum concentration of D in cholesterol of adult mice was 50% of that found in the body water when the animals were kept on D_2O for as long as 50–100 days.

Table 1. *Deuterium content of cholesterol obtained from tissues of animals whose body water was enriched with* D_2O *for 1–12 days between the* $(28-n)$*th and 28th day of pregnancy*

(D content of cholesterol (atom-% excess) expressed as the percentage of the D content (atom-% excess) of body water.)

No. of days on D_2O (n)	Cholesterol from			
	Maternal liver	Foetal placenta	Foetal liver	Foetal carcass
1	10·80	10·20	20·50	15·10
2	21·95	22·00	—	29·30
3	24·40	25·65	43·35	35·61
4	29·40	29·40	51·00	43·30
6	32·70	39·00	53·00	50·40
11	33·80	40·20	63·30	64·40
12	48·40	51·00	62·37	63·00

To test whether cholesterol in the foetus is synthesized throughout the development with a maximum isotope concentration of 63 atom-% excess, another experiment was carried out in which a pregnant rabbit was kept on D_2O for 7 days between the 16th and 23rd days of pregnancy. A 16-day rabbit embryo contains about 0·5 mg. and a 23-day foetus 12·5 mg. of cholesterol, i.e. 96% of the amount present on the 23rd day has accumulated between the 16th and 23rd days of intrauterine life. If the 12 mg. which have been added to the 0·5 mg. present in the 16-day embryo had been synthesized within the foetus with a D content of 63 atom-% excess, then the total foetal cholesterol should have a D content of 60·5 atom-% excess. The values found were 61·5 and 62 atom-% excess in foetal liver and carcass-cholesterol respectively and therefore are close to the prediction.

The D content of the cholesterol extracted from the placenta was only slightly more than that found for maternal liver cholesterol (Table 1). The ability of the foetal placenta to synthesize cholesterol was, however, proved definitely by results obtained after the injection of labelled acetate (Tables 5, 6). The low D content found may mean either a relatively slow turnover rate of cholesterol in the placenta, or, in addition to synthesis, absorption of cholesterol with low isotope content from the maternal blood. The absorption of cholesterol from the maternal blood and its storage by the placenta have previously been demonstrated (Popják, 1946). Since the D content of the cholesterol in the placenta was less than that in either the foetal liver or in the foetal carcass, the sterol in the latter tissues could not have been derived from the placenta, but must have been synthesized within the body of the foetus.

Synthesis of deuterium into glyceride and phospholipin fatty acids. The D content of glyceride and phospholipin fatty acids extracted from the maternal liver and foetal tissues is shown in Tables 2 and 3. The fatty acids of the foetal carcass were much richer in the isotope than those of the maternal liver, which proved the synthesis of fatty acids by the foetal tissues. The D content of the fatty acids extracted from the foetal liver and foetal placenta, however, exceeded the isotope content of the maternal liver fatty acids by only a narrow margin.

The interpretation of the results shown in Tables 2 and 3 is made somewhat difficult by the fact that these fatty acids are not homogeneous for it has been shown by Rittenberg & Schoenheimer (1937) that the saturated fatty acids are regenerated in the body much faster, and acquire a higher D content, than the unsaturated acids. It is known that the proportion of the saturated acids in the foetus is higher than in the adult animal (Chaikoff & Robinson, 1933). The results shown in Tables 2 and 3 might therefore be taken to indicate that the maternal saturated fatty acids, which should have a higher isotope content than the unfractionated acids, have been transferred selectively across the placenta and have accumulated in the foetal organs.

To decide if such an interpretation is valid, the glyceride fatty acids from the maternal and foetal liver and foetal carcass were fractionated into saturated and unsaturated acids. The D contents of the two fractions are shown in Table 4. The saturated fatty acids in the foetal carcass contained a higher concentration of isotope than the saturated acids of the maternal liver. In the experiment

recorded in the last line of Table 4, in which the body water of the animal was enriched with D_2O for 12 days, from the 16th to the 28th days of gestation, the D content of the saturated acids obtained from the foetal carcass was 50 atom-% excess. Rittenberg & Schoenheimer (1937) have shown that, during the total synthesis of saturated fatty acids in animals, 50 % of the stably bound H atoms in these acids are derived from the body water (see also Schoenheimer, 1941).

Table 2. *Deuterium content of glyceride fatty acids obtained from tissues of animals whose body water was enriched with* D_2O *for* 1–12 *days between the* $(28-n)$*th and* 28*th day of pregnancy*

(D content of fatty acids (atom-% excess) expressed as the percentage of the D content (atom-% excess) of body water.)

No. of days on D_2O (n)	Glyceride fatty acids from Maternal liver	Foetal placenta	Foetal liver	Foetal carcass
1	4·72	4·19	4·86	5·53
2	4·88	7·41	6·60	11·12
3	5·86	7·00	8·85	14·47
4	7·80	9·02	8·61	18·26
6	12·31	12·80	14·02	25·14
12	22·70	22·65	22·85	33·20

Table 3. *Deuterium content of phospholipin fatty acids obtained from tissues of animals whose body water was enriched with* D_2O *for* 1–12 *days between the* $(28-n)$*th and* 28*th day of pregnancy*

(D content of fatty acids (atom-% excess) expressed as the percentage of the D content (atom-% excess) of body water.)

No. of days on D_2O (n)	Phospholipin fatty acids from Maternal liver	Foetal placenta	Foetal liver	Foetal carcass
1	4·61	4·16	6·66	7·80
2	5·15	6·79	8·64	12·72
3	6·00	8·06	10·55	17·58
4	7·70	9·02	10·20	18·30
6	13·90	10·35	14·63	26·80
12	21·47	20·40	19·30	34·20

The D content of the saturated fatty acids in the foetal liver in the same experiment was, however, only 37·6 atom-% excess. If these fatty acids had been synthesized entirely in the foetal liver by the same biochemical mechanism as those in the foetal extrahepatic tissues, they might be expected to contain similarly 50 atom-% excess deuterium. One obvious explanation is that the fatty acids synthesized in the foetal liver with a high isotope content were diluted with fatty acids low in D from the maternal side. Another possible explanation will be discussed later.

Essentially similar considerations apply to the foetal unsaturated fatty acids. The unsaturated fatty acids in the foetal carcass had a higher isotope content than the corresponding acids in the maternal liver. Except for one experiment, the deuterium content of the unsaturated fatty acids in the foetal liver was, on the other hand, less than in the maternal liver. These results by themselves would therefore make it appear very doubtful that any of these unsaturated acids were synthesized within the foetal liver. It will be shown, with the aid of $CH_3{}^{14}COONa$, that there was definite synthesis of unsaturated acids in the foetal liver.

Fat synthesis in embryonal adipose tissue. It is generally assumed that in the adult animal the liver is the main site of fatty acid synthesis, the extrahepatic tissues being comparatively inert. Although results bearing on this problem, which will be reported separately, indicate that there are tissues in the adult animal which synthesize fatty acids even more rapidly than the liver, it is agreed that the adipose tissues are mainly stores of fat. In the foetal carcass most of the glyceride fat is present in adipose tissue which develops from the mesenchyme. Synthesis of glyceride fatty acids in the extrahepatic foetal tissues has already been demonstrated (Tables 3, 5), and it is thought that the embryonal fatty tissue is the principal organ of this synthesis. This suggestion is borne out by results shown in Table 5. These were obtained on the 23-day pregnant rabbit which was kept on D_2O for 7 days between the 16th and 23rd days of pregnancy, and which was in

Table 4. *Deuterium content of saturated and unsaturated glyceride fatty acids obtained from tissues of animals whose body water was enriched with* D_2O *for* 1–12 *days between the* $(28-n)$*th and* 28*th day of pregnancy*

(D content of fatty acids (atom-% excess) is expressed as percentage of D content (atom-% excess) of body water. The D content of the unfractionated glyceride fatty acids is shown in Table 2. S = saturated and U = unsaturated fatty acids.)

No. of days on D_2O (n)	Maternal liver S	Maternal liver U	Foetal liver S	Foetal liver U	Foetal carcass S	Foetal carcass U
1	7·60	4·10	6·36	4·10	8·20	3·59
3	6·08	5·59	12·00	7·50	21·64	7·31
4	—*	—*	12·60	5·60	26·80	7·70
6	17·89	9·36	22·90	8·00	39·20	17·02
12	33·40	19·14	37·60	12·00	50·00	31·70

* Insufficient material for fractionation.

Table 5. *D content (atom-% excess) and ^{14}C content of maternal and foetal fatty acids in a 23-day pregnant rabbit which was given D_2O for 7 days from the 16th to the 23rd day of pregnancy and was injected intravenously with 100 μc. of ^{14}C as $CH_3{}^{14}COONa$ 22 hr. before end of experiment*

(^{14}C content expressed as 1×10^{-5} μc./mg. substance.)

Source	Deuterium content of: Glyceride fatty acids	Deuterium content of: Phospholipin fatty acids	^{14}C content of: Glyceride fatty acids	^{14}C content of: Phospholipin fatty acids	^{14}C content of: Cholesterol
Maternal liver	5·3	9·44	2·53	2·66	8·16
Foetal liver	26·5	—*	18·36	14·64	32·40
Foetal carcass†	—*	36·7	13·15	12·64	20·00
Foetal adipose tissue‡	30·6	—	24·10	13·50	21·10
Foetal placenta	—*	—*	6·71	7·76	14·90

* Insufficient material for D determination.

† Denotes the body of the foetus without liver and adipose tissue which have been dissected out.

‡ This represents the earliest fatty tissue visible to the naked eye in the rabbit foetus and which develops as two distinct lobes over and between the scapulae; these fatty lobes continue forward on two sides of the neck which they surround like a collar.

addition injected with $CH_3{}^{14}COONa$ on the 22nd day. This experiment has already been mentioned in connexion with the foetal synthesis of cholesterol. The adipose tissue from the foetuses of this animal was dissected out and its fatty constituents analysed. The especially high degree of utilization of acetate for the synthesis of glyceride fatty acids in this tissue supports the view that the embryonal fatty tissue is one of the main sites of fat formation in the foetus. Unfortunately, sufficient material for fractionation of fatty acids into saturated and unsaturated fatty acids was not available from this experiment.

28th day of gestation and 16 hr. after the last injection of $CH_3{}^{14}COONa$. The ^{14}C content of the fatty acids and cholesterol was determined on the substances themselves with a thin mica-window Geiger-Müller counter as described in Appendix 2. The results of all seven experiments were qualitatively and quantitatively the same, and therefore the details of only one are shown in Table 6, where the ^{14}C contents of foetal cholesterol and fatty acids may be compared with those obtained from the maternal liver and plasma.

Whatever the mode of administration of the labelled acetate, the radioactivity of the maternal

Table 6. *^{14}C content of cholesterol and fatty acids in maternal and foetal tissues after intravenous injection of 50 μc. of ^{14}C as $CH_3{}^{14}COONa$ (4·3 mg.) in three doses during 24 hr.*

(^{14}C content expressed as 10^{-5} μc./mg. of substance. M = unfractionated fatty acids; S = saturated fatty acids; U = unsaturated fatty acids.)

Source	Cholesterol	Glyceride fatty acids: M	Glyceride fatty acids: S	Glyceride fatty acids: U	Phospholipin fatty acids
Maternal liver	8·0	1·39	1·83	0·36	1·11
Maternal plasma	6·5	—	—	—	—
Foetal placenta	11·1	2·2	—	—	2·33
Foetal plasma	19·0	—	—	—	—
Foetal liver	45·6	3·38	5·5	2·0	3·91
Foetal carcass	13·6	2·73	3·66	0·97	2·81

Utilization of acetate for cholesterol and fatty acid synthesis in the foetus. It has been shown by Bloch & Rittenberg (1942*a*, *b*) that acetate is utilized in animals for cholesterol and fatty acid synthesis.

Seven pregnant rabbits, six of which also received D_2O, were injected intravenously with $CH_3{}^{14}COONa$. The total radioactive dose was either 50 or 100 μc. of ^{14}C contained in 4·3 or 8·6 mg. of anhydrous sodium acetate. Either a single intravenous injection was given (as in the experiment shown in Table 5), or the total dose was divided into two or more evenly spaced injections over a period of 1–4 days. In each case the animals were killed on the

plasma cholesterol was always less than that of foetal cholesterol; hence (under our experimental conditions of repeated injections of the labelled precursor) the latter could not have been derived from the former. A comparison of the radioactivities of the cholesterol obtained from the foetal placenta, foetal plasma and foetal liver shows that the highly active cholesterol in the foetal liver was synthesized there and not transported with the foetal plasma from the placenta. These results then confirm in every respect those obtained with the aid of D and show the utilization of acetate C for cholesterol synthesis in the foetus as well as in the adult.

In two experiments the ^{14}C content of cholesterol and fatty acids extracted from the maternal placenta was also examined; they showed just detectable radioactivity (0·5–1·0 count/min. above background), corresponding to a ^{14}C content of less than 10^{-6} μc./mg. of substance. This finding indicates that, at least towards the end of pregnancy, the maternal placenta is metabolically rather inert. The observations suggest further that the source of plasma cholesterol, both in the adult and foetal animal, is the liver, since the ^{14}C content of the plasma cholesterol was closest to that of liver cholesterol both in the mother and the foetus.

Table 6 shows that the isotope content of the fatty acids in the foetal liver and carcass was about two or three times as high as in the maternal liver. Since the radioactivity of the fatty acids in the placenta was higher than in the maternal liver, but lower than in the foetal liver and carcass, it must be concluded that, just as in the case of cholesterol, the fatty acids in the foetal body were mainly synthesized there and not derived from either the placenta or the mother. The results show further that there was a higher percentage utilization of the carboxyl C of acetate for the synthesis of the saturated glyceride acids than for the synthesis of the unsaturated ones, which is a similar finding to that known to be the case for the adult animal (Rittenberg & Bloch, 1945).

Lipid synthesis in the mammary glands of pregnant rabbits. The material of this investigation gave also an opportunity to study the fatty acid and cholesterol metabolism of the developing but non-lactating mammary gland. We wish to record certain observations which form the basis of further experiments already in progress. Table 7 shows the D content of the fatty acids and cholesterol of the mammary glands of pregnant rabbits whose body water was enriched with D_2O for 1–12 days between the 16th and 28th days of pregnancy. As a comparison the values for the liver lipids are also shown in Table 1. It appears that there is synthesis of fatty acids and cholesterol in the mammary gland also, apart from any possible supplies of fatty materials from the liver via the blood stream. There is, however, an interesting feature, which becomes obvious when the data of Table 7 are plotted graphically (Fig. 1). The increases in D content of both phospholipin-fatty acids and cholesterol of the mammary gland show the usual smooth curves. In the case of the glyceride fatty acids, however, there is a sharp break, which appears between the 22nd and 24th days of pregnancy, i.e. between the experiments in which the animals were kept on D_2O for 6 and 4 days respectively. Since this discontinuity is not present in either the cholesterol or phospholipin-fatty acid curves, it cannot be ascribed to either experimental error or biological variations. A tentative explanation is offered, but requires further experimental work. The mammary glands of rabbits are usually well developed by the middle of the gestation period, at about the 16th day of pregnancy, when an active fat secretion begins. If the fats (chiefly glycerides) which accumulate between the 16th and 28th days of pregnancy have been chiefly synthesized by the gland of an animal given D_2O to drink, it is to be expected that the fatty acids should have a high D content. This appears to be the case for the last two experiments shown in Table 7. From the 24th

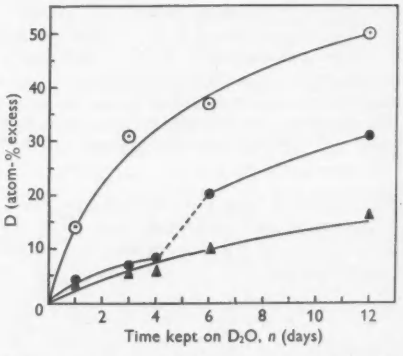

Fig. 1. Incorporation of deuterium into cholesterol and glyceride and phospholipin fatty acids of mammae of non-lactating pregnant rabbits. Cholesterol, ⊙—⊙; glyceride fatty acids, ●—●; phospholipin fatty acids, ▲—▲.

Table 7. *Deuterium content of glyceride and phospholipin fatty acids and of cholesterol in mammary glands and liver of pregnant rabbits whose body water was enriched with* D_2O *for* 1–12 *days between the* $(28-n)$*th and* 28*th day of pregnancy*

(D content of lipids (atom-% excess) expressed as the percentage of the D content (atom-% excess) of body water.)

No. of days on D_2O (n)	Mammary gland			Liver		
	Glyceride fatty acids	Phospholipin fatty acids	Cholesterol	Glyceride fatty acids	Phospholipin fatty acids	Cholesterol
1	4·51	3·59	14·26	4·72	4·61	10·8
3	7·00	5·23	31·40	5·88	6·00	24·4
4	8·40	5·60	—	7·80	7·70	29·4
6	20·48	10·13	37·40	12·31	13·90	32·7
12	31·30	16·28	50·00	22·70	21·47	48·4

day onwards (the first three experiments in Table 7), however, the very active fat synthesis must have slackened, since the D content of the glyceride fatty acids increased only slightly between the 24th and 28th days of pregnancy. It is probable that the lack of emptying of the gland retarded fat synthesis in the mammary gland and that the fat which had already accumulated did not participate in active metabolic processes.

In Table 8 are recorded the ^{14}C contents of the fatty acid fractions obtained from the mammary glands of two animals injected with $CH_3{}^{14}COONa$. Qualitatively, the results were the same in all seven experiments, but the amounts of ^{14}C synthesized into the fatty acids of the mammary gland showed much greater variations from animal to animal than in the liver. This might be expected in an organ fulfilling only a temporary function and being greatly influenced by hormonal and other factors.

Table 8. ^{14}C *content of glyceride and phospholipin fatty acids in mammary glands of non-lactating pregnant rabbits injected intravenously with* $CH_3{}^{14}COONa$

(^{14}C content expressed as 1×10^{-5} μc./mg. of fatty acid carbon. In Exp. 1, two equal injections of the labelled acetate were given daily for 4 days; the total ^{14}C dose was 50 μc. (4·3 mg. of acetate). In Exp. 2, two injections, 50 μc. each, were given 6 hr. apart and the animal was killed 20 hr. after the 1st injection.)

	Exp. 1	Exp. 2
Glyceride fatty acids		
Total fatty acids, unfractionated	10·2	42·0
Non-volatile fatty acids:		
Unfractionated	5·1	9·9
Saturated	3·4	7·2
Unsaturated	5·9	10·2
Volatile fatty acids*:		
Water soluble (chiefly butyric)	38·1	184·5
Water insoluble (chiefly caproic)	52·6	136·0
Phospholipin fatty acids	2·91	5·7

* We are indebted to Mr T. H. French of the National Institute for Research in Dairying, University of Reading, for the separation of the volatile acids by steam distillation.

The most important feature of the results shown in Table 8 is the appreciably higher radioactivity of the mixed glyceride fatty acids (volatile + non-volatile) as compared with that of the non-volatile acids. This is accounted for by the much greater isotope content of the short-chain volatile acids (C_4–C_8). It has already been reported (Popják, Folley & French, 1949) that the short-chain volatile acids, which are characteristic components of the milk fat of the herbivores, and which were separated from the mixed glyceride acids of these rabbits, contained 7–18 times more ^{14}C than the non-volatile acids. These results confirm the suggestion of Folley & French (1949) that acetate is utilized for the synthesis of short-chain fatty acids of milk fat. The alternative mechanism for the formation of these fatty acids in milk, by the degradation of long-chain acids suggested by Hilditch (1947), if it occurs, must be of lesser importance.

The non-volatile saturated acids of the mammae (Table 8) contained much less ^{14}C than the unsaturated acids; a notable difference from other organs, e.g. liver, where the reverse occurs. The interpretation of this fact is open to question at present. The results obtained in two animals, in which a comparison could be made with the liver fatty acids, indicated definite synthesis of unsaturated fatty acids in the mammary gland. The low ^{14}C content of the saturated glyceride fatty acids in the breasts might be explained by a relative resting state of the glands before lactation begins, or by assuming that some of the saturated acids (chiefly palmitic) were derived from the blood stream. It should be also borne in mind that the glyceride fatty acid samples were undoubtedly contaminated by connective tissue fat, which cannot be completely removed from the mammary glands. It is not certain what proportion of the fat extracted from the mammae was within glandular secretory cells, or stored in the ducts, or in the interlobular adipose tissue. The presence of the short-chain volatile acids, however, indicates that the material represented primarily fat secreted by the mammae.

DISCUSSION

Cholesterol and fatty acid synthesis in the foetus. The qualitative interpretation of the results presented does not seem to offer serious difficulties. These may be summarized briefly. It has been shown that the foetal liver and the extrahepatic tissues of the foetus are able to synthesize both cholesterol and fatty acids from small molecules and that acetate is a source of carbon in these syntheses just as in the tissues of the adult animal. It appears that no significant amount of cholesterol in the foetus is derived from the preformed maternal compound. The placenta, while able to synthesize cholesterol and fatty acids, also absorbs significant amounts of these substances, or their compounds, from the maternal blood stream (cf. Popják, 1946; Popják & Beeckmans, 1950).

It seems very probable that most of the fatty acids (at least the saturated ones) found in the extrahepatic tissues of the foetus have been synthesized there. The fatty acids in the foetal liver, on the other hand, are derived probably from two sources: (*a*) from synthesis within the organ, and (*b*) from maternal substances transmitted through the placenta. But it is remarkable that the saturated fatty acids do not appear to pass in appreciable quantity from the foetal liver to the rest of the foetal body. It is recalled that in the experiment in which

the animal was given D_2O for 12 days between the 16th and 28th days of pregnancy, the saturated glyceride fatty acids of the foetal carcass had a D content of 50 % and in the foetal liver only 37·6 % of that present in the body water. It may be mentioned in this connexion that, between the 20th and 28th days of pregnancy in the rabbit, the foetal liver becomes extremely fatty, containing occasionally as much as 20 % of neutral fat (Popják, 1946). It is tempting to ascribe this fatty change to infiltration by maternal fat transported across the placenta.

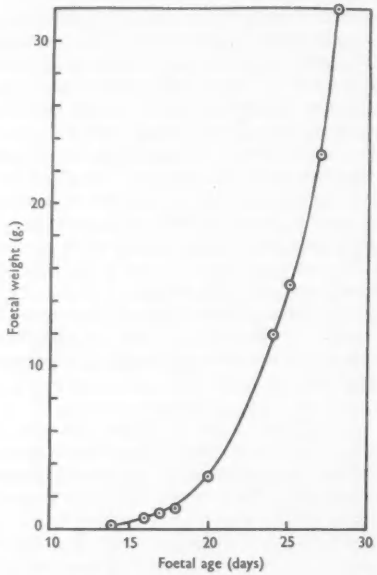

Fig. 2. Growth of rabbit foetus from the data of Boyd (1935) and own observations.

Goldwater & Stetten (1947) concluded that approximately 10·7 % of the foetal cholesterol in the rat was derived from the labelled cholesterol which had been fed to the mother. From the results of this investigation it is inferred that no significant amount of cholesterol in the rabbit foetus could have been derived from maternal sources. This conclusion will be further supported by quantitative assessment of our results. It seems probable that the differences between the results of this investigation and those of Goldwater & Stetten (1947) are due to differences between species. In one experiment in which they gave D_2O to a rat during the whole period of gestation, the D content of the foetal cholesterol was 55 % of that in the body water. In our experiments we found a concentration of about 63 % when the rabbits were given D_2O to drink between the 16th and 23rd or 16th and 28th days of pregnancy.

Calculation of rate of synthesis and rate of degradation of a substance in a growing system from isotope experiments. An important problem in the dynamics of foetal-fat metabolism is to what extent foetal tissues can degrade the fatty substances. To answer this question a quantitative assessment of the results obtained with the aid of D will be necessary.

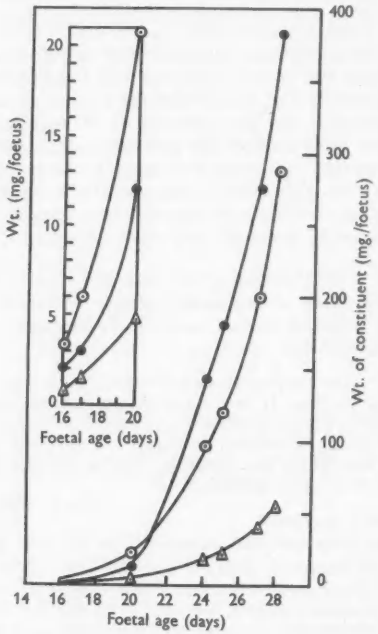

Fig. 3. Accumulation of cholesterol and glyceride, and phospholipin fatty acids in the rabbit foetus. The points give the total amounts of fatty substances present in the foetus between the 16th and 28th days of intrauterine life. The inset on the left shows on a larger scale the quantities present between the 16th and 20th days. Values calculated from data of Boyd (1935) and own observations. Cholesterol, △—△; phospholipin fatty acids, ⊙—⊙; glyceride fatty acids, ●—●.

In the adult animal, in a state of metabolic equilibrium, the rate of synthesis of a compound must be equal to its rate of degradation. In the developing embryo, which shows the phenomenal growth rate illustrated in Fig. 2, this concept of dynamic equilibrium cannot be true; neither can one speak of 'turnover rate' in the manner applied to adult animals; instead two terms, the rate of synthesis and the rate of degradation of a compound must be computed. Fig. 3 shows the accumulation of cholesterol and of fatty acids in the rabbit foetus compiled from the data of Boyd (1935) and our own determination.

The calculations which follow are based on principles similar to those applied by Goldwater & Stetten (1947) to foetal glycogen.

Let dA/dt = the rate of accumulation of substance A in the tissues; db/dt = the rate of synthesis of substance A; $d\alpha/dt$ = the rate of degradation of substance A; then

$$\frac{dA}{dt}=\frac{db}{dt}+\frac{d\alpha}{dt}. \quad (1)$$

Let z = total amount of isotope present in substance A at time t; $A(t)$ = total amount of substance A at time t; then the isotope concentration (i) in the substance at time t is

$$i=\frac{z}{A(t)}. \quad (2)$$

Let i_m = the maximum isotope concentration which can be attained in the compound. If it be assumed that the newly synthesized molecules have an isotope concentration of i_m and the molecules which are being degraded the same isotope concentration (i) as the substance as a whole in the tissues, then the rate of change in z may be written

$$\frac{dz}{dt}=\frac{i_m\,db}{dt}+\frac{i\,d\alpha}{dt}; \quad (3)$$

since

$$i=\frac{z}{A(t)},$$

$$\frac{di}{dt}=\frac{1}{A(t)}\frac{dz}{dt}-\frac{z}{[A(t)]^2}\frac{dA}{dt}; \quad (4)$$

By substituting in (4) the value of dz/dt from (3) and the value of dA/dt from (1), we obtain

$$\frac{di}{dt}=\frac{1}{A(t)}(i_m-i)\frac{db}{dt} \quad \text{or} \quad \frac{db}{dt}=\frac{di}{dt}\frac{A(t)}{i_m-i}. \quad (5)$$

There is no general solution to equation (5), but it may be evaluated empirically by graphical solution of di/dt. It can be shown graphically (Fig. 4) that

$$\frac{di}{dt}=\frac{i_m-i}{\Delta t},$$

and hence substituting in (5)

$$\frac{db}{dt}=\frac{A(t)}{\Delta t}. \quad (6)$$

It should be pointed out that the value of Δt in equation (6), depending as it does on a number of factors, is not the same throughout the growth period of the foetus. For this reason the rates of accumulation, synthesis and degradation have been calculated for the 28th day of pregnancy only, although the experimental data are adequate for similar calculations to be carried out for other foetal ages also.

The data shown in Fig. 3 permit the calculation of dA/dt and the isotope experiments yield db/dt and therefore $d\alpha/dt$ (equation 1) may also be computed. In Figs. 5–8 the continuous curves show the accumulation of the fatty constituents in the foetal tissues. These curves are the same as those shown in Fig. 3 except that they are plotted in reverse, i.e. from the 28th day to the 16th. This was necessary, since this was the time dimension of the isotope experiments with D. The total amount of the substance present in the foetal tissues on any particular day is given in these figures by $(A_{28}-y)$. The left-hand ordinates, y, give the total amount of substance which had accumulated in the foetus between the $(28-n)$th and

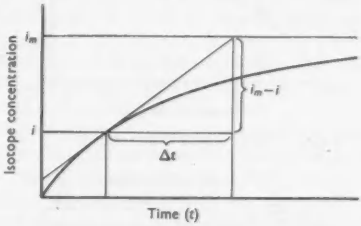

Fig. 4. Graphical evaluation of di/dt, where i = isotope concentration in substance, i_m = maximum isotope concentration attainable in substance. The curve is an assumed isotope concentration-time curve with $i \to i_m$. The procedure is merely to determine the slope of the tangent to this curve.

28th days. For example, the value of y at 22-day foetal age gives the amount of either cholesterol or fatty acids which had accumulated between the 22nd and 28th days of gestation (not the total amount of substance present on the 22nd day, which is given by $(A_{28}-y)$). dA/dt (the rate of accumulation of the substance) on any particular day is given by $(A_{28}-y)/\Delta t'$. The isotope-concentration-time curves (interrupted lines with circles) have been superimposed in Figs. 5–8 on such a scale that the experimentally determined value of i_m (the isotope concentrations found in foetal substances in the 12-day experiment) coincides with the point of A_{28}.

The simple qualitative interpretation of the relationships shown in Figs. 5–8 is that if the points of the isotope-concentration-time curve fall below the curve representing the rate of accumulation of the particular substance, there has been a dilution with substances of lower isotope concentration than i_m. If the points of the isotope-concentration-time curve, on the other hand, are above the curve of accumulation, the substance is being degraded as well as synthesized.

The rates of accumulation (dA/dt), synthesis (db/dt) and destruction ($d\alpha/dt$) of cholesterol and fatty acids have been calculated from the data

shown in Figs. 5–8 for the 28-day-old rabbit foetus. The values are shown in Table 9. The accuracy of these calculations and the deductions drawn from them rest on the correctness of the shape of the curves giving the rates of accumulation of the fatty substances in the foetus; the absolute values of the co-ordinates of these curves are of less importance. Unfortunately, the rates of accumulation cannot be obtained for the same foetuses on which the isotope determinations were carried out, and mean values had to be used from the data of Boyd (1935) and from our own observations for this purpose. The interpretation of the data shown in Table 9 is therefore given with the above reservations.

Figs. 5–8. Graphical evaluation of rate of accumulation (dA/dt), synthesis (db/dt) and degradation ($d\alpha/dt$) of fatty substances in foetus with the aid of equations:

$$dA/dt = db/dt + d\alpha/dt \quad \text{and} \quad db/dt = A(t)/\Delta t.$$

(See text p. 555.)

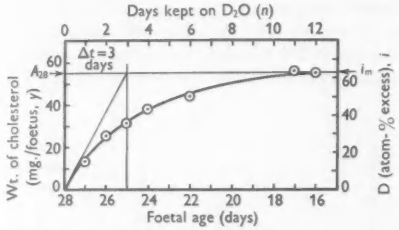

Fig. 5. Foetal carcass cholesterol.

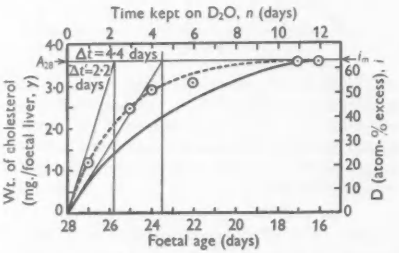

Fig. 6. Foetal liver cholesterol.

It appears that the rates of accumulation and synthesis of foetal carcass cholesterol are equal, and therefore there is no destruction of cholesterol by the extrahepatic foetal tissues. In the foetal liver, however, the values obtained for cholesterol were:

$$db/dt = 1{\cdot}64 \text{ mg./day/liver (2·4 g.)}$$

and

$$d\alpha/dt = -0{\cdot}82 \text{ mg./day/liver.}$$

Thus one-half of what was synthesized on the 28th day was degraded or removed from the foetal liver, resulting in a net accumulation of 0·82 mg. It should be clear from these data that the 55 mg. of cholesterol present in the foetal carcass on the 28th day of gestation could not have been obtained from the

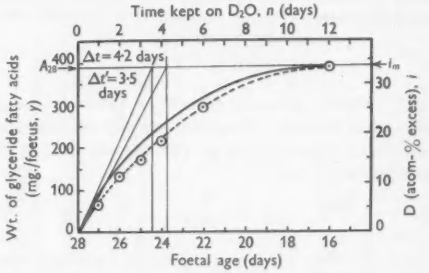

Fig. 7. Foetal carcass glyceride fatty acids.

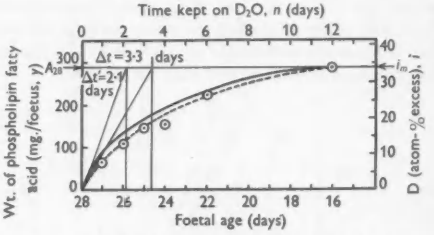

Fig. 8. Foetal carcass phospholipin fatty acids.

Table 9. *Values of rates of accumulation* (dA/dt), *synthesis* (db/dt) *and degradation* ($d\alpha/dt$) *of cholesterol and fatty acids calculated from data shown in Figs. 5–8 for 28-day-old rabbit foetuses*

$$dA/dt = db/dt + d\alpha/dt. \quad (1)$$

Source and substance	Wt. of tissues on 28th day (g.)	Total amount of substance present in tissues, A_{28} (mg.)	$\Delta t'$ (days)	Δt (days)	dA/dt (mg./day)	db/dt (mg./day)	$d\alpha/dt$ (mg./day)
Foetal carcass	32	—	—	—	—	—	—
Cholesterol	—	55	3	3	18·3	18·3	0
Glyceride fatty acids	—	389	3·5	4·2	111·2	92·7	+18·5
Phospholipin fatty acids	—	287	2·1	3·3	136·6	87·0	+49·6
Foetal liver	2·4	—	—	—	—	—	—
Cholesterol	—	3·6	4·4	2·2	0·82	1·64	−0·82

foetal liver, even if all the cholesterol synthesized in the latter organ during the entire pregnancy had been transferred to the extrahepatic tissues.

The quantitative interpretation of the results on synthesis of foetal fatty acids is more difficult than in the case of cholesterol. The chief obstacle in the way of precise calculations is the heterogeneity of the fatty acids. It has already been pointed out that in the extrahepatic tissues of the foetus the chief source of both saturated and unsaturated fatty acids is endogenous synthesis. A calculation of rates of synthesis and destruction for the saturated and unsaturated fatty acids separately would be permissible only if the rates of accumulation of the two types of acids in the foetus were known during growth. Such data, however, are not available; only the rates of accumulation of the mixed glyceride and phospholipin fatty acids are known, as shown in Figs. 7 and 8. However, if it be assumed that the ratio of the two types of fatty acids in the foetal carcass is the same throughout the pregnancy and that the newly synthesized mixed glyceride and phospholipin fatty acids contain an average of 33·2 and 34·2 atom-% excess deuterium, respectively, then the figures shown in Table 9 are obtained. The positive values obtained for the rate of destruction ($d\alpha/dt$) of fatty acids indicate that on the 28th day of pregnancy, in addition to synthesis of deuterio-fatty acids, there was a source of non-isotopically labelled fatty acids (or of fatty acids of low isotope content). The most probable source is the maternal plasma. It seems likely that the fatty acids obtained by the foetal carcass from the mother are chiefly the unsaturated acids. This is supported by the observation that when the D concentration of the saturated acids from the foetal carcass is plotted against time of D_2O administration, the shape of the curve obtained approximates the curve showing the rate of accumulation of fatty acids. However, inspection of the figures shown in Table 4 reveals that the D content of the unsaturated glyceride fatty acids in the foetal carcass increased linearly with time, which is quite unusual. This type of curve might result from the placental transfer of maternal unsaturated fatty acids of low isotope content, as already inferred. Naturally these fatty acids would have to traverse the foetal liver first before they can reach the extrahepatic tissues of the foetus. Since the passage of whole phospholipin molecules across the placenta could not be demonstrated (Popják & Beeckmans, 1950), the mechanism of placental transfer of fatty acids remains an open question.

Some differences may exist between foetal liver and the extrahepatic tissues in respect of the mechanism of fatty acid synthesis. This is strongly suggested by the wide discrepancy between the results obtained with the aid of D and of ^{14}C. It will be recalled that in animals given D_2O to drink, the D content of the fatty acids in the foetal carcass was much greater than in the foetal liver. In experiments with labelled acetate, however, the fatty acids in the foetal carcass contained slightly less ^{14}C than in the liver (except for the adipose tissue glyceride fatty acids from the 23-day-old foetus, Table 5) (cf. Tables 2 and 6). If dilution by maternal fat were the sole explanation for the relatively low D content of the foetal-liver fatty acids, one might expect this dilution to be apparent with ^{14}C also. This was not the case. It seems possible that the fatty acid precursors in the extrahepatic tissues of the foetus are more highly oxidized than those in the foetal liver and hence during fatty acid synthesis, which must involve reduction, more D is being introduced in the former than in the latter. It has also been suggested recently by Bloch (1948) in another connexion, that a mechanism of fatty acid synthesis may exist not involving the hydrogen of the body water. This problem is now being investigated.

Reduced rates of, or absence of, degradation of cholesterol and of fatty acids in the foetus appear to be of particular significance. First, as a general biological phenomenon, such an observation may indicate that growth is associated not so much with increased synthesis as with an inhibition of degradation. Rittenberg, Sproul & Shemin (1948) also concluded that the growth of the regenerating liver, after partial hepatectomy, is the result of the inhibition of degradative processes, since the rate of protein synthesis in the regenerating liver was not significantly faster than in normal animals. Secondly, the absence of apparent degradation specifically of fatty acids must mean that the primary energy source in foetal metabolism is carbohydrate. The results of Goldwater & Stetten (1947) are of particular interest in this connexion. These authors have calculated the rate of synthesis and degradation of glycogen in the rat foetus. They found that about 500 mg. of glycogen are synthesized/100 g./day in the 18·75-day-old foetus; of this, however, only about 200 mg. were deposited, the rest being degraded. The amount degraded could have been utilized for energy, or fat synthesis, or both.

SUMMARY

1. The synthesis of cholesterol and fatty acids in the rabbit foetus has been demonstrated by the rapid incorporation of deuterium from the body water into these substances and by the utilization of ^{14}C-labelled acetate for these syntheses.

2. It is concluded (*a*) that all foetal cholesterol is obtained by synthesis within the body of the foetus; (*b*) that while the foetal extrahepatic tissues synthesize cholesterol, they do not degrade it; and (*c*) that the foetal liver synthesizes as well as degrades cholesterol, but the amount degraded is only one-

half of the amount synthesized on the twenty-eighth day of pregnancy.

3. Synthesis of both saturated and unsaturated fatty acids in the foetal liver and extrahepatic tissues (embryonal adipose tissue) has been demonstrated. The fatty acids in the foetal liver contained much less deuterium than those in the extrahepatic tissues; this difference might be due to dilution of fatty acids in the foetal liver by maternal substances. However, when ^{14}C-labelled acetate was given, the fatty acids in the foetal liver contained more ^{14}C than in the foetal carcass. The discrepancy in the results obtained with deuterium and ^{14}C suggests that the fat precursors in the foetal liver and extrahepatic tissues are not identical.

4. The foetal placenta synthesizes all lipids studied, but it also absorbs these from the maternal circulation. The maternal placenta appears metabolically inert, at least towards the end of pregnancy.

5. A method, based on principles developed by Goldwater & Stetten (1947), is presented for the calculation, in isotope experiments, of the rate of accumulation, synthesis and degradation of a substance in a growing system.

6. Since there was no apparent destruction of fat in the foetus, it is inferred that the primary source of energy in the foetus must be carbohydrate and that growth is characterized not so much by increased rate of synthesis as by reduced rate or absence of degradation.

7. The synthesis of cholesterol and fatty acids in the non-lactating mammae of pregnant rabbits has been shown. Evidence for the specific utilization of acetate for the formation of C_4–C_8 acids in the mammae is also presented (see also Popják *et al.* 1949).

We are indebted to Mr D. O'Connor for the mathematical formulation presented in the discussion, to Mr D. Hart for his skilful technical assistance and to Mr F. Crisp for the artificial insemination of the rabbits. The ^{14}C used in this investigation was obtained from the U.S. Atomic Energy Commission; the labelled acetate was synthesized from $Ba^{14}CO_3$ at the Radiochemical Centre, Amersham, Bucks.

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APPENDIX 1

Water Bath suitable for the Maintenance of Temperatures to within $\pm 1{\cdot}5 \times 10^{-3}$ °. Micropipette for the Accurate Delivery of Small Volumes

By G. POPJÁK

Water baths suitable for the determination of deuterium in water samples by the falling-drop method are not available commercially in this country. While such baths have probably been constructed in many laboratories, descriptions are not readily available. The author has been requested from several quarters to describe the bath used in the preceding investigation and which can be constructed by very simple means. No originality is claimed.

Dimensions and materials of bath: 30 × 30 × 50 (height) cm.; galvanized-copper sheet. The bath was made with plate-glass windows at the front and back, and the whole placed in a wooden box. The 2·5 cm. space between the walls of the bath and the wooden box was filled with suitable lagging material. The glass windows were left uncovered.

Thermostat. This was a toluene-Hg thermostat constructed from approximately 5 yards of thin-walled Pyrex

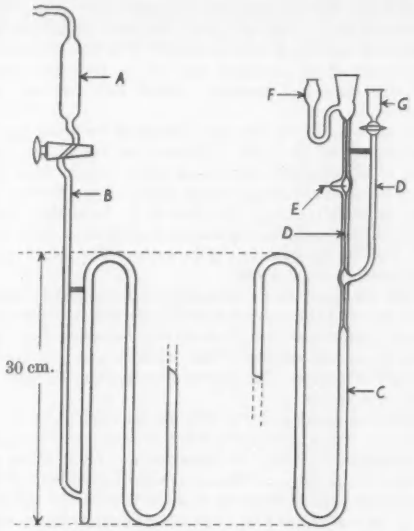

Fig. 1. Diagram of toluene-Hg thermostat made of Pyrex-glass tubing and used in bath for determination of D_2O by the falling-drop method. *A*, Hg reservoir for coarse adjustment of Hg level; *B*, capillary tubing of 3 mm. bore; *C*, glass tubing, 10 mm. internal diameter; *D*, capillary tubing of 1·5 mm. bore; *E*, tungsten wire sealed into capillary; *F*, benzene reservoir; *G*, small Hg reservoir for fine adjustment of Hg level.

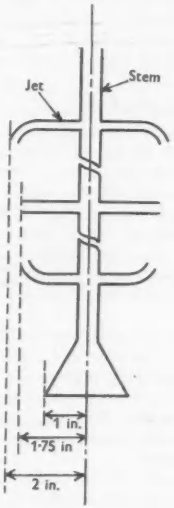

Fig. 2. Diagram of centrifugal stirrer. The stem is made of 0·5 in. (12·5 mm.) brass tubing, the jets of 0·25 in. (6 mm.) copper tubing and the funnel of thin copper sheet.

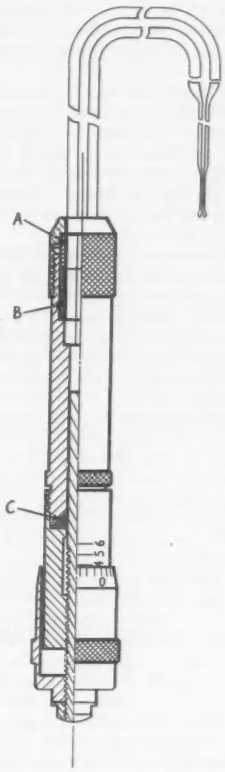

Fig. 3.

Fig. 4.

Fig. 3. Micropipette for the delivery of small drops. *A*, metal ring which compresses rubber sleeve, *B*, placed around the glass tubing; *C*, neoprene gland washer which provides air-tight seal between piston and barrel. Other parts are self-explanatory. Scale approx. 2/3.

Fig. 4. The drawn-out end of the glass part of the micropipette. The diagram illustrates how the correct shape of the tip is obtained: first the tubing is drawn out into a stout capillary which is drawn out further over a microflame. The arrows indicate the line where the tip should be cut; the tip is then ground around with a dentist's drill fitted with a fine carborundum disk. Water drops delivered hang from such tips.

tubing of 10 mm. internal diameter. The glass tubing was bent in ∽ shapes and was placed on three sides of the bath. The thermostat head was of the usual design. Fig. 1 illustrates the thermostat diagrammatically. The thermostat was operated with a Sunvic electronic relay and Proportioning Head. The temperature of the bath can be maintained for months within $\pm 1{\cdot}5 \times 10^{-3}°$ with this regulator at 27°. The *heating element* was bare Nicrome wire wound on glass tubing and distributed on three sides of the bath at the bottom; the total resistance of the heating element was 4 Ω draining 3 amp. at 12 V. No trouble was encountered on account of lack of insulation of the heating element at this low voltage. Ordinary distilled water was used in the bath.

The stirrer was a centrifugal type driven by a small 24 V. motor (see Fig. 2). This stirrer was found very efficient without causing troublesome vibrations. A drop of permanganate placed under the funnel of the stirrer appears through the top jets within a few seconds and stains the water of the bath uniformly in about 10 sec. No difference in temperature at the top and bottom of the bath could be detected with a Beckmann thermometer.

Maintenance of bath. In the last instance this bath has been in constant operation day and night for 1 year without change of water. No preservative was added to the water, but a layer (about 1 cm. thick) of liquid paraffin was poured over the surface. The anaerobic condition suppressed completely the growth of moulds, etc. Before the use of liquid paraffin the water had to be changed about every month in spite of various preservatives added.

It was found that a layer of benzene (free of S compounds) between the Hg column and contact needle prevented oxidation of the surface of Hg and ensured a clean contact and smooth running of the thermostat. For the continuous replacement of this benzene the cup of the thermostat head was connected through a small side arm with a reservoir.

The micropipette used for the delivery of the water drops was designed by Dr B. W. Robinson (see Fig. 3). It consisted of a calibrated micrometer screw which moved a piston in a snugly fitting barrel filled with mercury. A Pyrex capillary (1·5 mm. bore) bent in ∩ shape and drawn out at the end into a fine tip was fixed air tight to the metal parts with an expandable rubber washer. The metal parts were made of stainless steel.

When the tip of the Pyrex capillary has the correct shape shown in Fig. 4 the water drops delivered under the surface of the *o*-fluorotoluene hang from the tip, otherwise the drops creep up on the outside of the capillary and lead to inaccuracy of delivery. The pipette assembly was clamped in a rack and pinion.

While the construction of this pipette requires precision machining it has great advantages over the one described by Keston, Rittenberg & Schoenheimer (1937–8) as no suction is required when filling. It can be filled with water sufficient for the delivery of at least three drops and can easily be rinsed between successive determinations with 2–3 cu.mm. of the new water sample. The accuracy of delivery is better than 1% in the region of 10 cu.mm. The water drops are delivered under the surface of the *o*-fluorotoluene and are detached by the surface tension of the latter on raising the pipette.

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APPENDIX 2

Preparation of Solid Samples for Assay of ^{14}C

By G. POPJÁK

All radioactivity measurements reported in the previous investigation were carried out on solid samples (cholesterol, cholesterol-digitonide, fatty acids, Pb salts and Na salts of fatty acids). 'Infinite thickness' samples, i.e. 25 mg. of material/sq.cm. were prepared on disks of identical geometry. The measurements were carried out with a He-filled bell-shaped Geiger-Müller counter which had a thin mica window. Under our conditions of counting a solid sample which contained 10^{-3} μc. of ^{14}C/mg. of substance gave approximately 1800 counts/min. in an 'infinite thickness' sample of 2 sq.cm. area. The background of our apparatus was 8–10 counts/min. The calibration of the counter was carried out with a sample containing known amounts of ^{14}C.

When sufficient material is available, as in the investigation described, assay of ^{14}C in 'infinite thickness' samples has the great advantage that the radioactive counts obtained on the various samples are linearly proportional to the specific activities and hence the ^{14}C contents may be expressed without self-absorption corrections. Further, the assay of ^{14}C directly on organic compounds of high C content avoids the undesirable dilution when the organic compounds are first combusted and the CO_2 obtained is collected as $BaCO_3$. The assay of ^{14}C on organic materials has the added advantage that the samples may be stored in common atmosphere, whereas with $Ba^{14}CO_3$ special measures are required to avoid exchange with atmospheric CO_2.

Several methods have been described for the preparation of solid samples for the assay of ^{14}C (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949) to suit individual requirements. The technique to be described has been used in this Institute for the assay of ^{14}C in a variety of crystalline and amorphous compounds (apart from those named above) and found very satisfactory.

Mounting material. Disks made of plastic material (Perspex or Polythene) and moulded to the shape shown in Fig. 1*a* were used. Two types were employed: (i) with solid, and (ii) with perforated, bottom.

Preparation of samples on solid disks ('pellet' technique). The solid disks were used for preparation of 'infinite thick-

ness' samples from solid substances (amorphous and crystalline) when plenty of material was available. Somewhat more than the weight required to give 'infinite thickness' of the finely powdered substance was first spread evenly over the area of the disk with a spatula, then the material was compressed into a pellet with a stainless steel press (Fig. 1*b*), which just fitted into the sample area of the disk. The edge of the disk was wiped clean while the press was still in position. When samples of sticky material (e.g. cholesterol, cholesteryl acetate and some Na salts of fatty acids) are being prepared, the press is gently rotated before lifting off the disk; this prevents the pellet getting stuck on to the metal. With other substances, like $BaCO_3$, cholesterol-digitonide, haemin, choline derivatives, Pb salts of fatty acids, the above precaution is not necessary. Samples of free fatty acids (solids) may be prepared by first smearing the material with a spatula as evenly as possible over the entire surface of the disk, and then levelling off any unevenness by the mere touch of a microflame (from a glass capillary jet) without melting the whole sample. Disks of two sizes were used: (1) with a sample area of 2·0 sq.cm.; and (2) with a sample area of 1·2 sq.cm. The ratio of counts obtained with these two disks was exactly 2:1·2. These sample areas are much smaller than those used by many American workers, who also employ counters with windows of 5–7·5 cm. diameter. Our counter had a window diameter of only 2·33 cm. The low background count (8–10 counts/min.) of such a counter is a good recommendation for its use.

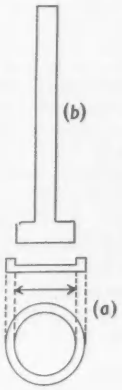

Fig. 1.

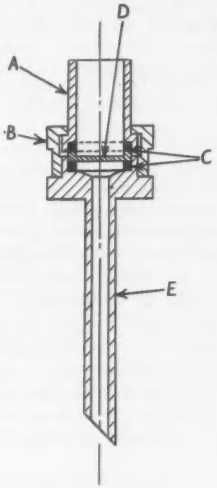

Fig. 2.

Fig. 1. (*a*) Shape of plastic disks used for preparation of samples for assay of ^{14}C. (*b*) Stainless steel press used for 'pellet' technique.

Fig. 2. Small demountable Büchner funnel made of stainless steel for collection of precipitates for assay of ^{14}C. *A*, funnel; *B*, ring for clamping funnel to lower portion; *C*, neoprene washers; *D*, perforated plastic filter disk fitted with filter paper; *E*, tube to fit into filter flask.

Preparation of samples on perforated disks. The perforated disks, which had the same dimensions as the solid ones, were used with a filter assembly shown in Fig. 2. Filter paper, punched slightly larger than the sample area of the disk, was first pressed into the disk with the tool shown in Fig. 1*b*. The disk, thus prepared, after being dried and weighed was clamped into the filter. The whole assembly is equivalent to a small demountable Büchner funnel from which the filter pad with the assay material can be taken out and placed under the counter. The substance to be assayed (e.g. cholesterol-digitonide, $BaCO_3$, etc.) was first ground in a test tube with a glass rod to a fine powder and suspended in a suitable medium (ethanol, ethanol-ether, methanol, or any other medium in which the compound and sample holder were insoluble). The suspension was then transferred into the filter and first allowed to settle for 1–2 min., then suction was applied. When the suspension medium was filtered through, the funnel of the filter assembly was removed and the suction continued under an infrared lamp until the material became dry. The disk then could be removed and the ^{14}C assayed. The counts obtained on duplicate samples usually agreed within 2–3%, and with the 'pellet' technique within the statistical error of counting.

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Glycogen Formation and Glucose Uptake of Isolated Muscle with 11-Deoxycorticosterone and 11-Dehydro-17-hydroxycorticosterone

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The isolated diaphragm of the rat produces glycogen from glucose in oxygenated Ringer solution, especially in the presence of insulin (Gemmill, 1940; Hechter, Levine & Soskin, 1941; Stadie & Zapp, 1947; Corkill & Nelson, 1947). This glycogenesis is inhibited by the addition of small doses of deoxycorticosterone (Verzár & Wenner, 1948*a*) during rest and during work (Mentha, Vögtli & Verzár, 1948). The isolated muscle takes up glucose from the solution (Krahl & Park, 1948; Perlmutter & Greep, 1948) and this also is inhibited by deoxycorticosterone (Leupin & Verzár, 1949). Other steroids were found to have varying effects. The inhibition of glycogen formation (expressed as decrease of glycogen production in mg./100 g. muscle) with 5 mg./100 ml. deoxycorticosterone (DOC) was 136, with corticosterone 66, with 17-hydroxycorticosterone 44 and with 10 mg./100 ml. dehydrocorticosterone acetate 92. Thus DOC proved to be the most active in this respect (Verzár & Wenner, 1948*b*).

It is generally thought that DOC, in contrast to 11-oxygenated corticosteroids, has no action on carbohydrate metabolism in the living animal. We have, however, shown that in the adrenalectomized, otherwise intact, animal, DOC also, if given over a longer period, restores liver and muscle glycogen to normal values (Montigel & Verzár, 1943; Sass-Kortsák, Wang & Verzár, 1949). But there is no doubt that the 11-oxygenated corticosteroids have a more pronounced action, especially in short-term experiments (Olson, Jacobs, Richert, Thayer, Kopp & Wade, 1944; Wang & Verzár, 1949*a*).

The corticosteroid which is most active on carbohydrate metabolism is 11-dehydro-17-hydroxycorticosterone acetate (compound E). Since this was now to hand in large enough quantities, we have done experiments to compare the activity of this compound with that of DOC on the isolated diaphragm. The adrenal cortical extract of the Upjohn Co. was also available for comparison.

EXPERIMENTAL

The method of Gemmill (1940, 1941) was used with slight modifications. The diaphragm of rats was quickly removed after pithing, cut into three parts and weighed on a torsion balance. The middle (vertebral) part was used for the estimation of the initial glycogen content. The other two parts were placed in vessels with 2 ml. of Ringer solution, containing phosphate buffer (Hastings, Muus & Bessey, 1939). Glucose (200 mg.) and 1 i.u. of insulin (Lilly)/100 ml. were added. All experiments lasted 90 min. The muscle was shaken in O_2 at 37°. The controls described in Table 1 show that the three parts of the diaphragm have equal glycogen contents and therefore can be used in parallel experiments, as was also done by Verzár & Wenner (1948*a*) and Mentha *et al.* (1948).

Table 1. *Glycogen content of the diaphragm of the rat*

(Vertebral part = middle part.)

	Glycogen content (mg./100 g.)			Glycogen content of side parts as percentage of content of vertebral part	
No.	Vertebral part	Side part I	Side part II	Side part I	Side part II
1	120	170	130	142	108
2	178	182	191	102	107
3	171	131	126	77	74
4	194	194	157	100	81
5	176	121	110	69	62
6	155	125	160	81	103
7	180	154	167	86	93
8	118	100	148	85	125
9	169	184	169	109	100
			Mean ± S.E.M.	95 ± 7*	95 ± 7*

* These values show that the glycogen contents of the two side parts do not differ significantly from one another or from that of the vertebral part.

Deoxycorticosterone or its acetate and compound E were dissolved in that amount of ethanol which would give a final ethanol concentration of 0·5 ml./100 ml. Ethanol in this concentration does not influence glycogen formation (Verzár & Wenner, 1948*a*). The solubility of 11-dehydro-17-hydroxycorticosterone acetate in Ringer solution is very low. Prof. T. Reichstein, to whom we are grateful for help in this matter, informed us that saponification gives a more soluble product. Saponification was done with $KHCO_3$ in methanol-water. About one-third of the hydrolysate was crystalline compound E with m.p. 213–217° (E_1). The residue (E_2) was amorphous and contained an unknown quantity of compound E together with an unidentified steroid. E_1 had a solubility similar to that of deoxycorticosterone; E_2 was easily soluble in water. We used E acetate, E_1 and E_2 and also a mixture of E_1 and E_2 in the original proportion of 1:2, and the action of these was compared with that of deoxycorticosterone and its acetate.

Glycogen estimations were carried out as in earlier papers (Verzár & Wenner, 1948*a*). Glucose was determined, following deproteinization according to Folin (1919), by the method of Hagedorn & Jensen (1923).

Normal and adrenalectomized rats of 100–200 g. body weight, from the Institute's stock, were used throughout.

RESULTS

Inhibition of glycogen formation

Normal animals. In Table 2 are shown experiments with the addition of DOC and in Table 3 (series II–VII) experiments with compound E. In Table 4 the mean values of all the experiments on normal animals are collected. In Table 4 the initial glycogen contents are not given and 'glycogen formation' is the difference between the glycogen content before and after incubation for 90 min. in oxygenated Ringer solution with 200 mg. glucose and 1 i.u. insulin/100 ml., calculated for 100 g. muscle. 'Glucose uptake' is the difference in glucose content in the same solution at the beginning and the end of a 90 min. period, also calculated for 100 g. muscle.

but there is still an almost equally large glucose uptake which obviously has nothing to do with glycogen formation. This glucose might be used for energy production. Thus it seems that the quantity of DOC used inhibits only the glycogen formation, and so a correspondingly smaller amount of glucose is taken up.

Glycogen formation was decreased by E_1 from 324 to 276, by E_2 from 307 to 210, by the $E_1 + E_2$ mixture from 257 to 187 and the E acetate from 228 to 188 mg./100 g. muscle (Table 4). For comparison DOC acetate was tested. It decreased glycogen formation from 286 to 159 mg./100 g. muscle.

Table 4 contains all mean values from the above series. Whilst it is clear that DOC inhibits glycogen formation and glucose uptake, Compound E was only about one-third of the activity of deoxycorticosterone. The number of experiments is not large enough to distinguish between the activity of the single E fractions. The glucose uptake does not show regular differences. DOC acetate has a smaller action on isolated muscle than has DOC itself. It was for this reason that we formerly used the latter in all experiments on isolated muscle. In Tables 3 and 4 some experiments with adrenal cortical extract (Upjohn Co.) are also shown. No inhibition was found.

Small doses of DOC and compound E tested on diaphragm of normal animals. In all previous experiments on the isolated muscle only an inhibition of glycogen formation has been observed. In the intact animal on the contrary glycogen production is increased with DOC and compound E. However, recently we have been able to show that DOC in large doses, which have in addition an anaesthetic action, decreases glycogen production in the intact animal, just as with the isolated diaphragm (Wang & Verzár, 1949*b*). We therefore tested again whether it might be possible to increase glycogen production in isolated muscle with much smaller quantities of

Table 2. *Glycogen formation and glucose uptake by isolated diaphragm muscle of normal animals without and with addition of* 11*-deoxycorticosterone* (5 *mg./100 ml.*) *and in the presence of insulin*

(Exp. no. 1–31.)

	Initial glycogen (mg./100 g.)	Glycogen formation (mg./100 g.)		Glucose uptake (mg./100 g.)	
		Without DOC	With DOC	Without DOC	With DOC
Mean and S.D.	285±23	279±17	58±8	453±24	195±23

It is obvious that the addition of 5 mg. of DOC/100 ml. gives an almost complete inhibition of glycogen formation (from 279 to 58 mg./100 g. muscle), while the glucose uptake is inhibited for a much lesser degree (from 453 to 195 mg./100 g. muscle). The absolute decrease in glucose uptake is nearly identical with the decrease in glycogen formation,

these hormones. In a few experiments of Verzár & Wenner (1948*a*) with smaller doses either inhibition or no action was found. It seemed possible that the latter could be the result of a balancing of glycogenetic and glycogenolytic activity.

We therefore tested the action of small quantities of DOC (0·01–0·1 mg./100 ml.) and of compound E

Table 3. *Glycogen formation and glucose uptake by isolated diaphragm muscle of normal animals without and with different corticosteroids in the presence of insulin*

(DOC acetate, deoxycorticosterone acetate. E acetate, 11-dehydro-17-hydroxycorticosterone (compound E). E_1, crystalline fraction of hydrolysate of compound E. E_2, amorphous fraction of hydrolysate of compound E. E_1+E_2, E_1+E_2 (1:2).)

Exp. no.	Addition (mg./100 ml.)	Initial glycogen (mg./100 g.)	Glycogen formation (mg./100 g.)		Glucose uptake (mg./100 g.)	
			Without addition	With addition	Without addition	With addition
32	E_1, 5	186	410	356	550	460
33	E_1, 5	142	—	330	—	357
34	E_1, 5	197	—	400	—	500
35	E_1, 5	217	313	233	—	390
36	E_1, 5	228	—	246	—	280
37	E_1, 5	166	—	210	—	460
38	E_1, 5	85	—	201	—	395
39	E_1, 10	199	249	201	485	340
40	E_1, 10	167	—	373	—	550
41	E_1, 10	246	—	208	—	215
42	E_2, 5	90	307	252	415	408
43	E_2, 5	85	—	163	—	408
44	E_2, 5	225	—	217	—	410
45	E_1+E_2, 5	495	135	120	200	370
46	E_1+E_2, 5	208	212	212	308	334
47	E_1+E_2, 10	450	84	32	70	290
48	E_1+E_2, 10	214	360	244	—	—
49	E_1+E_2, 10	145	189	82	340	133
50	E_1+E_2, 15	141	426	249	609	504
51	E_1+E_2, 15	114	284	103	195	330
52	E_1+E_2, 15	124	190	170	170	170
53	E acetate, 5	148	86	95	360	390
54	E acetate, 5	166	207	61	225	150
55	E acetate, 5	149	306	256	270	375
56	DOC acetate, 5·6	229	351	178	380	210
57		202	163	28	369	145
58		40	260	210	325	350
59		91	370	218	300	300
60	Adrenal cortical extract (0·1 ml./100 ml.)	243	187	187	450	375
61		253	257	283	360	360
62		175	140	130	220	250
63		300	150	170	250	360
64		250	200	180	450	400
65	Adrenal cortical extract (0·5 ml./100 ml.)	260	222	230	360	340
66		210	210	250	310	315
67		205	235	200	322	277

Table 4. *Mean values of glycogen formation and glucose uptake by isolated diaphragm muscle of normal animals with different corticosteroids in the presence of insulin (series I–VII)*

(Number of animals in brackets. For abbreviations see Table 3.)

Series	Addition (mg./100 ml.)	Glycogen formation (mg./100 g.)		Glucose uptake (mg./100 g.)	
		Without addition	With addition	Without addition	With addition
I	DOC, 5	279 (31)	58 (31)	453 (31)	195 (31)
II	E_1+E_2, 5–15	257 (7)	187 (9)	305 (7)	311 (8)
III	E_1, 5–10	324 (3)	276 (10)	517 (2)	394 (10)
IV	E_2, 5	307 (1)	210 (3)	415 (1)	408 (3)
V	E acetate, 1–5	228 (9)	188 (9)	285 (3)	305 (3)
VI	DOC acetate, 5·6	286 (4)	159 (4)	344 (4)	251 (4)
VII	Adrenal cortical extract (0·1–0·5 ml./100 ml.)	200 (8)	186 (12)	340 (8)	347 (8)

(0·05–0·5 mg./100 ml.) (series VIII and X). For comparison we also, at the same time, repeated experiments with larger inhibitory doses (series IX and XI). DOC inhibited glycogen production at 0·5 mg./100 ml. and above, and compound E at 1 mg./100 ml. and above. Table 5 shows the mean values. The *P* (probability) values (after Fisher) for the difference of glycogen production with or without addition are also shown. Whilst it is obvious that larger doses of DOC inhibit glycogen production in series IX, it is possible, from the mean values of series VIII on thirty-eight single experiments, that small doses of DOC, from 0·01 to 0·1 mg./100 ml., may somewhat increase glycogen production in muscle.

For compound E the slight increase of glycogen production with 0·05–0·5 mg./100 ml. in series X and also the inhibition with large doses of 1·0–5·0 mg./100 ml. (series XI) has only the small *P* value of about 0·5.

Adrenalectomized animals. The same experiments were then done with the diaphragms of adrenalectomized animals. It seemed possible that the glycogenetic action might be more obvious in the muscle of such animals. Table 6 shows the mean values. Compound E gave, in doses between 1·0 and 5·0 mg. (series XIII), only a slight inhibition, and, in smaller doses of 0·1–0·5 mg. (series XII), a slight increase of glycogen production. No difference was large enough to be significant.

Verzár & Wenner (1948*a*, *b*) had already shown that large doses of DOC (5 mg./100 ml.) inhibit glycogen production in the muscle of adrenalectomized animals. These experiments were therefore not repeated, but in series XIV twelve experiments with small concentrations of DOC (0·01–0·05 mg./100 ml.) are shown. No significant increase of glycogen production could be detected.

DISCUSSION

In continuation of the work of Verzár & Wenner (1948*a*, *b*) and Mentha *et al.* (1948), in which it was found that DOC inhibits glycogen formation in isolated muscle, it has now been shown that DOC also inhibits the uptake of glucose to an extent which corresponds to the decrease of glycogen formation. We thus confirm the observations of Perlmutter & Greep (1948) and those of Krahl & Park (1948) that the glucose uptake of a normal diaphragm in Ringer solution is about twice as great as the equivalent of the glycogen produced in the same period. DOC probably does not diminish the amount of glucose taken up and used directly for energy production.

The inhibition of glycogen production by 11-dehydro-17-hydroxycorticosterone (compound E), either as acetate or saponified, was about one-third of that found with deoxycorticosterone; and no regular inhibition of the uptake of glucose was observed.

Thus the corticosteroid 11-dehydro-17-hydroxycorticosterone, which has the greatest influence on glycogen production in the normal or adrenalectomized animal, has a much smaller inhibitory action on glycogenesis in the isolated muscle than has deoxycorticosterone. This is in agreement with the earlier result of Verzár & Wenner (1948*b*) that various 11-oxygenated corticosteroids have a much smaller inhibitory action than DOC. This 'inhibition of glycogen formation' may be rather an acceleration of glycogen breakdown in the isolated muscle. This interpretation would lead to the view that the smaller action of deoxycorticosterone on glycogen production in the living animal is explicable by a relatively stronger action of this substance on glycogen breakdown. The 11-oxygenated corticosteroids on the contrary might have less action in increasing

Table 5. *Mean values of glycogen formation of normal animals in the presence of insulin (series VIII–XI)*

Series	Addition (mg./100 ml.)	No. of animals	Initial glycogen (mg./100 g.)	Glycogen formation (mg./100 g.) Without addition	Glycogen formation (mg./100 g.) With addition	*P* values for the difference without and with addition
VIII	DOC, 0·01–0·1	38	152±13	228±21	264±18	0·2–0·3
IX	DOC, 0·5–5·0	9	159±29	144±11	47±25	0·01
X	Compound E_2, 0·05–0·5	23	168±14	237±21	254±20	0·5–0·6
XI	Compound E_2, 1·0–5·0	9	129±13	223±29	182±34	0·3–0·4

Table 6. *Mean values of glycogen formation of diaphragm of adrenalectomized animals in presence of insulin (series XII–XIV)*

Series	Addition (mg./100 ml.)	No. of animals	Initial glycogen (mg./100 g.)	Glycogen formation (mg./100 g.) Without addition	Glycogen formation (mg./100 g.) With addition	*P* values for the difference without and with addition
XII	Compound E, 0·1–0·5	5	269±42	232±51	238±39	0·9–1·0
XIII	Compound E, 1·0–5·0	4	253±39	243±37	198±46	0·4–0·5
XIV	DOC, 0·01–0·05	12	208±20	254±26	262±23	0·8–0·9

glycogenolysis and have a greater effect on glycogenesis.

We have been impressed by another difference between these two corticosteroids. DOC acetate (10 mg.) injected intraperitoneally into a rat of 100–150 g. produces (as first shown by Selye, 1941*a*, *b*, *c*) a narcosis-like state which lasts for several hours, whereas 10 and even 20 mg. of compound E, injected into similar animals, have no narcotic action whatever. Also, in concentrations above 5 mg./100 ml. in Ringer solution, DOC decreases the work obtainable from the isolated diaphragm muscle (Mentha *et al.* 1948). Compound E showed no such action up to 10 mg./100 ml.

The inhibition of work through DOC might be due to the same cellular process as the narcosis in intact animals. Lately (Wang & Verzár, 1949*b*) we have been able to demonstrate an inhibition of glycogenesis also in intact animals, using doses of DOC large enough to cause narcosis.

We have tried, but failed, to show in a decisive way that very small doses of either DOC or of compound E have a glycogenetic effect on the isolated diaphragm either of normal or of adrenalectomized rats. If such an activity does exist, as series VIII and X on normal and XII and XIV on adrenalectomized rats seem to suggest, the action is very slight and cannot therefore be decisively proved. However, it might be that if such a slight action is continuous in the intact animal it leads to the glycogenesis which is observed there.

SUMMARY

1. Deoxycorticosterone, in concentrations of 5 mg./100 ml. or above, inhibits the glycogen formation of the isolated diaphragm of normal rats in the presence of 200 mg. glucose and 1 i.u. insulin/100 ml.

2. Deoxycorticosterone also inhibits that part of the glucose uptake which corresponds to the glycogen formation. About half of the original glucose uptake remains unaffected.

3. 11-Dehydro-17-hydroxycorticosterone (compound E) decreases glycogen formation only about one-third as much as deoxycorticosterone, and the effect on glucose uptake is variable.

4. Small doses of DOC or of compound E which have no inhibitory action on glycogen formation cause a very slight increase of glycogen production in normal and also in adrenalectomized animals. This effect is, however, so small that it cannot be considered significant.

5. Possible ways are discussed of correlating the different activities of these corticosteroids on the carbohydrate metabolism of isolated muscle with their different activities in the intact animal.

We wish to express our thanks to Merck and Co., Rahway, N.J., for the compound E, to Dr D. Ingle of Upjohn Co., Kalamazoo, U.S.A., for the adrenal cortical extract and Ciba A. G., Basel, for the deoxycorticosterone used in these experiments. The work was done with the assistance of the Swiss Federal Foundation for Scientific Production.

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The Influence of Corticosteroids on Deamination in the Surviving Diaphragm

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The surviving isolated diaphragm of the rat takes up glucose and produces glycogen. These processes are inhibited by deoxycorticosterone (Verzár & Wenner, 1948*a*, *b*) and also by 11-dehydro-17-hydroxycorticosterone (Leupin & Verzár, 1950). It seemed possible that changes in the protein metabolism under the influence of the added corticosteroids might lead to a change in the rate of deamination of amino-acids. The ammonia production in the diaphragm of normal pithed rats in the presence and absence of corticosteroids was therefore studied.

METHODS

The animal was killed and the diaphragm divided into two equal parts; these were suspended in 2 ml. of phosphate-buffered oxygenated Ringer solution at 37° with the addition of 200 mg. glucose/100 ml. of fluid and 1 i.u. insulin/100 ml. as in earlier experiments. One half of the diaphragm was used without addition and the other with the addition of a corticosteroid. In other experiments a different corticosteroid was added to each half of the muscle. All additions were made in ethanolic solution as previously described (Leupin & Verzár, 1950).

Measurement of ammonia production. After 90 min. incubation the NH_3 content was estimated by the method of Conway & O'Malley (1942) and Conway (1947). The solution (0·5 ml.) was deproteinized by Folin's method and then transferred to the outer chamber of a Conway vessel together with 1 ml. of saturated K_2CO_3. The inner chamber of the Conway vessel contained 1 ml. of 1% (w/v) boric acid in 20% (v/v) ethanol with bromocresol green-methyl red indicator. The vessels were greased with a hard fat instead of

Table 1. *Ammonia production by diaphragm with and without the addition of various corticosteroids*

Exp. no.	Diaphragm without addition of corticosteroid. NH_3 produced (mg./100 g. of muscle)	Diaphragm with addition of corticosteroid: Compound added (mg./100 ml.)	Diaphragm with addition of corticosteroid: NH_3 produced (mg./100 g. of muscle)
1	8·1	DOC, 4·4	14·7
2	4·3	DOC, 4·4	4·7
3	22·5	DOC, 5	7·4
4	6·1	DOC, 5	7·4
5	5·8	DOC, 5	7·3
6	6·5	DOC, 5	4·7
7	6·1	DOC, 5	7·3
8	18·0	DOC, 5	10·9
9	10·4	DOC, 5	19·7
10	9·4	DOC, 5	10·4
11	7·6	DOC, 5	8·2
12	10·0	DOC, 5	11·9
13	7·8	DOC, 5	8·2
14	7·4	DOC, 5	8·0
15	2·9	DOC, 5	9·6
16	12·6	DOC, 5	11·8
17	6·1	DOC, 5	6·3
18	12·0	DOC, 5	7·9
19	9·0	DOC, 5	10·1
20	7·7	DOC, 5	7·7
21	10·3	DOC, 5	8·1
22	3·7	DOC, 7	3·9
23	6·6	DOC, 7	4·3
24	4·0	DOCA, 5	14·1
25	7·2	DOCA, 5·6	6·2
26	9·6	DOCA, 5·6	11·3
27	11·5	DOCA, 5·6	10·6
28	4·0	DOCA, 5·6	10·2
29	8·9	DOCA, 5·6	6·7
30	7·2	Compound E, 5	7·2
31	10·7	Compound E, 5	14·0
32	8·3	Compound E, 5	15·1
Mean	8·5 ± 2·2		9·2 ± 2·0

Table 1 (*cont.*)

In most of the following experiments the effects of two different corticosteroids on the two halves of the same diaphragm were compared:

	Diaphragm with addition of corticosteroid		Diaphragm with addition of corticosteroid	
Exp. no.	Compound added (mg./100 ml.)	NH_3 produced (mg./100 g. of muscle)	Compound added (mg./100 ml.)	NH_3 produced (mg./100 g. of muscle)
33	DOCA, 5	8·4	E, 5	8·7
34	DOCA, 5	17·2	E, 5	11·6
35	DOCA, 5	7·2	E, 5	6·3
36	DOC, 5	12·2	E_1, 5	21·8
37	DOC, 5	10·5	E_1, 5	10·3
38	DOC, 5	11·9	E_1, 5	28·0
39	DOC, 5	6·5	E_1, 5	8·9
40	DOC, 5	9·3	E_1, 5	9·3
41	None	13·3	E_1, 10	9·8
42	None	9·9	E_2, 5	5·6
43	E_1, 5	25·0	E_2, 5	10·6
44	DOC, 5	10·8	E_2, 5	10·0
45	None	9·0	E_1+E_2, 15	5·6
46	DOC, 5	8·0	E_1+E_2, 15	8·3
47	DOC, 5	7·8	E_1+E_2, 15	9·7
48	E_1, 10	8·7	DOC, 5	11·5
	Mean	**10·9**$\pm$0·3		**11·0**$\pm$2·1

vaseline. The reaction time was 4 hr. Titration was with 0·002 or 0·001 N-HCl from a horizontal Conway burette. The NH_3 values in Table 1 are given as mg. NH_3/100 g. muscle.

RESULTS

Table 1 shows the results of thirty-two experiments in which ammonia production was measured with and without addition of corticosteroids, and also of sixteen experiments in which different corticosteroids were applied to the two halves of the muscle. DOC represents deoxycorticosterone and DOCA its acetate. Compound E is the acetate of 11-dehydro-17-hydroxycorticosterone; E_1 is the crystalline fraction and E_2 the amorphous residue, obtained by saponifying compound E. E_1+E_2 is a mixture of E_1 and E_2 in the proportion 1:2 (Leupin & Verzár, 1950).

The mean value of ammonia production was 8·5 mg. NH_3/100 g. muscle in experiments in which no addition was made to the Ringer solution; the corresponding value in experiments in which DOC (4·4–7·0 mg./100 ml.) or DOCA and compound E (in similar concentrations) were added was 9·2 mg. The mean values for the experiments in which different corticosteroids were added to two halves of the same muscle were 10·9 and 11·0 mg. NH_3/100 g. muscle respectively. Values for the two halves of the same muscle generally agreed fairly well as shown in the second series and no significant effects of the different corticosteroids on ammonia production were observed. However, ammonia figures for different muscles even without addition of steroids showed large individual variations and extreme values as low as 3·7 mg. and as high as 28 mg./100 g. muscle were observed.

DISCUSSION

No effect of the addition of various corticosteroids on the ammonia production by isolated muscle has been observed. The inhibition of glycogen production from glucose does not involve an increased deamination of amino-acids. This negative result with isolated surviving muscle must of course not be applied to the intact animal.

SUMMARY

1. The surviving diaphragm of the rat incubated in oxygenated Ringer solution containing 200 mg. glucose and 1 i.u. insulin/100 ml. produces about 8·5 $\pm$ 2·2 mg. of ammonia/100 g. muscle in 90 min.

2. Addition of deoxycorticosterone and 11-dehydro-17-hydroxycorticosterone did not alter the ammonia production.

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Lipids of Rabbit Blood Cells. Data for Red Cells and Polymorphonuclear Leucocytes

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For a number of years chemists have been interested in the lipids of both red and white blood cells. Many of these early observations have been reviewed by Bloor (1943). Recent publications from this laboratory have been concerned with the chemistry of white blood cells, particularly the enzymes of the polymorphonuclear leucocyte of the rabbit (Cram & Rossiter, 1949; Rossiter & Wong, 1949, 1950; Haight & Rossiter, 1950). It seemed of interest to investigate the lipids in these cells by the micro methods already in use (Johnson, McNabb & Rossiter, 1948*a*, *b*; 1949*a*, *b*, *c*). At the same time observations were made on the lipids of the rabbit red cells.

Many workers have investigated the lipids of blood cells, but few have attempted to distinguish between the different phospholipins. Exceptions are the reports of Thannhauser & Setz (1936*b*) on ox red cells, Erickson, Williams, Bernstein, Avrin, Jones & Macy (1938) on red-cell stroma, and Kirk (1938), Williams, Erickson, Avrin, Bernstein & Macy (1938), Erickson, Avrin, Teague & Williams (1940) and Hack (1947) on human red cells. As far as we are aware, no attempt has been made to determine the distribution of phospholipins in white cells.

METHODS

Red cells. Blood was withdrawn from the marginal ear vein of the rabbit. Heparin was used as an anticoagulant as recommended by Boyd & Murray (1937). The blood was centrifuged at 3000 rev./min. and the plasma and the buffy-coat layer aspirated off. The red cells were pipetted from the bottom of the packed cell layer to avoid contamination with white cells, which are considerably richer in lipids. The complete analysis in duplicate could be done on 7–8 g. packed cells.

Polymorphonuclear leucocytes. Polymorphonuclear leucocytes were obtained from the peritoneal cavity of the rabbit by the method of de Haan (1918), the details of which have already been described by Cram & Rossiter (1949). Usually 12 rabbits were injected at the same time and the resulting white cells pooled. These cells, 95% of which were polymorphonuclear leucocytes, were concentrated by centrifuging, washed with 0·9% (w/v) NaCl, packed at 3000 rev./min. in a tared centrifuge tube and allowed to drain for 30 min. The tube was then re-weighed. By this means 2–5 g. packed cells could be obtained, sufficient for the complete analysis in duplicate. No attempt was made to control the age, sex, weight or the dietary condition of the animals.

Analytical methods. The lipids were extracted from the cells as described by Johnson *et al.* (1948*a*). The concentration of cerebroside (or glycosphingoside), total and free cholesterol, total phospholipin, monoaminophospholipin (or phosphoglyceride), lecithin (phosphatidyl choline) and total fatty acid was determined for each sample. From these figures the concentration of ester cholesterol, sphingomyelin (phosphosphingoside), kephalin, neutral fat and total lipid was calculated. Details of the analytical procedures, together with a discussion of their specificity and accuracy are given in papers by Johnson *et al.* (1948*a*, 1949*c*). In the absence of information concerning the mean molecular weights of the fatty acids in the various lipid components of blood cells, the assumptions made previously for the lipids of the nervous system were used as a basis for the calculations. It is known that the fatty acids of the sphingomyelin from both red blood cells (Thannhauser, Setz & Benotti, 1938) and spleen (Thannhauser & Boncoddo, 1948) are different from those of brain sphingomyelin. The same is probably true for the fatty acids of the other lipid components of both red and white cells. The general nature of the results would not, however, be altered appreciably if the mean molecular weights of either the sphingolipins or the phosphoglycerides of blood cells were slightly different from those that have been arbitrarily assumed.

RESULTS

Table 1 gives the mean value and the standard error of the mean for the cerebroside, free, total and ester cholesterol, total phospholipin, total fatty acid, neutral fat and total lipid of both the red cells and the polymorphonuclear leucocytes of the rabbit. Table 1 also gives the value for each lipid component expressed as a percentage of the total lipid, and the ratio of the value found for the polymorphonuclear leucocytes to that for the red cells.

The concentration of total lipid in the polymorphonuclear leucocytes (1764 ± 110 mg./100 g.) was much greater than that in the red cells (484 ± 19 mg./100 g.). The polymorphonuclear leucocytes contained more of each of the lipid components investigated. The greatest difference was in the figures for neutral fat. Whereas the polymorphonuclear leucocytes contained 530 ± 130 mg./100 g., the red cells contained negligible quantities (41 ± 18 mg./100 g.). The polymorphonuclear leucocytes also contained over three times as much total phospholipin

Table 1. *Lipids of rabbit blood cells*

(Results as mg./100 g. fresh tissue.)

	Red cells				Polymorphonuclear leucocytes				
	No. of observations	Mean	S.E. of mean	Percentage of total lipid	No. of observations	Mean	S.E. of mean	Percentage of total lipid	Ratio white cells/ red cells
Cerebroside	6	33	8	6·8	6	41	30	2·3	1·24:1
Free cholesterol	6	146	3	30·2	7	234	10	13·3	1·60:1
Total cholesterol	6	146	3	30·2	7	243	10	13·8	1·66:1
Ester cholesterol	6	0	0	0	7	9	2	0·5	—
Total phospholipin	6	264	8	54·5	7	950	40	53·9	3·60:1
Total fatty acid	6	230	18	47·6	7	1140	100	64·6	4·96:1
Neutral fat	6	41	18	8·5	7	530	130	30·2	12·9:1
Total lipid	6	484	19	100·0	7	1764	110	100·0	3·65:1

Table 2. *Phospholipins of rabbit blood cells*

(Results as mg./100 g. fresh tissue.)

	Red cells				Polymorphonuclear leucocytes				
	No. of observations	Mean	S.E. of mean	Percentage of total phospho-lipin	No. of observations	Mean	S.E. of mean	Percentage of total phospho-lipin	Ratio white cells/ red cells
Total phospholipin	6	264	8	100·0	7	950	40	100·0	3·60:1
Monoaminophospholipin	6	212	9	80·4	7	670	30	70·6	3·16:1
Lecithin	6	94	5	35·6	7	300	30	31·6	3·19:1
Sphingomyelin	6	52	2	19·6	7	280	10	29·4	5·29:1
Kephalin	6	118	7	44·8	7	370	20	39·0	3·14:1

(950 ± 40 mg./100 g.) as did the red cells (264 ± 8 mg./100 g.). The concentration of cerebroside was similar in each type of cell, but the polymorphonuclear leucocytes had slightly more total cholesterol. Although there was no measurable ester cholesterol in the red cells, there was a small, but definite, concentration of ester cholesterol in the polymorphonuclear leucocytes.

The relative distribution of the various lipids in each of the cell types was also different. Neutral fat accounted for only 8·5 % of the total lipid of the red cells, whereas for the polymorphonuclear leucocytes the figure was 30·2 %. The percentage of the total phospholipin was similar for each cell type, but the percentage of cholesterol and cerebroside was somewhat greater in the red cells.

Table 2 gives the mean value and the standard error of the mean for each of the individual phospholipins, together with the value for each individual phospholipin expressed as a percentage of the total phospholipin. The ratio of the value found for the polymorphonuclear leucocytes to that for the red cells is also given in Table 2. As seen previously, there was over three times the concentration of total phospholipin in the polymorphonuclear leucocytes as there was in the red cells. Each of the three individual phospholipins was increased, but the greatest difference was for sphingomyelin. Whereas the white cells contained 280 ± 10 mg./100 g., the concentration in the red cells was only 52 ± 2 mg./100 g. This difference is also seen when the individual phospholipins are expressed as a percentage of the total phospholipin. The percentage of sphingomyelin in the polymorphonuclear leucocytes was much greater, while for the red cells the percentage of kephalin was increased.

DISCUSSION

The rabbit polymorphonuclear leucocytes were obtained from sterile peritoneal exudates by the method of de Haan (1918). Although microscopic examination showed that these cells appeared similar morphologically to those circulating in normal rabbit blood, the possibility that they may differ from normal cells, either physiologically or chemically, cannot be excluded. An outstanding feature of the results is that these cells contained much more lipid than rabbit red cells. The greatest difference was in the concentration of neutral fat, but they contained more of each of the lipid constituents studied. In the red cells, kephalin was the phospholipin present in greatest concentration, while in the polymorphonuclear leucocytes there was relatively less kephalin and more sphingomyelin.

The figures obtained for cholesterol, total phospholipin, total fatty acid and total lipid of the red cell are essentially similar to those reported in the literature. When the diversity of the methods used by previous workers is considered, it is remarkable that the agreement is so good. Iscovesco (1912), Mayer & Schaeffer (1913), Horiuchi (1920) and Bloor (1921) have given figures for the red cells of the rabbit, while similar figures for the red cells of man have been given by Mayer & Schaeffer (1913), Bloor (1916), Iwatsuru (1924), Boyd (1936*a*) and Erickson, Williams, Hummel & Macy (1937).

Bürger & Beumer (1913) reported that the stroma of red cells contained sphingomyelin, an observation confirmed by Thannhauser & Setz (1936*b*), using the reineckate precipitation method developed by Thannhauser and his colleagues (Thannhauser & Setz, 1936*a*; Thannhauser & Benotti, 1938). Subsequently sphingomyelin was isolated from the stroma of ox red cells by Thannhauser *et al.* (1938). It is now known that the reineckate precipitation procedure for the determination of sphingomyelin is unreliable (Hack, 1946; Schmidt, Benotti, Hershman & Thannhauser, 1946), so the finding of Thannhauser & Setz (1936*b*), that up to 50 % of phospholipin of red cells is sphingomyelin, is probably an overestimate. However, Erickson *et al.* (1940), using a micro modification of Thannhauser's reineckate method, reported that sphingomyelin accounted for 16 % of the phospholipins of the red cells of man. Hunter (1942), also using the reineckate method, reported a figure of 26 % for the red cells of the cat. Kirk (1938), determining sphingomyelin as ether-insoluble phospholipin, found that 24 % of the total phospholipin of the red cells of man was sphingomyelin, while Hack (1947), employing the method Schmidt *et al.* (1946) used in our study on the rabbit, reported a value of 25 %. For the rabbit the figure was 20 %.

The relatively high concentration of kephalin in red cells was commented upon by Bürger & Beumer (1913) and Haurowitz & Sládek (1928) who failed to detect any choline after the saponification of the ether-soluble phospholipins of red cells. The high content of kephalin has been reported for the red cells of man by Kirk (1938), Williams *et al.* (1938), Erickson *et al.* (1940) and Hack (1947). Similar figures are now reported for the red cells of the rabbit.

Few workers have reported on the concentration of cerebroside in red cells. We constantly found a substance in the red cells of the rabbit which was estimated as cerebroside, but the limitations of the method, discussed previously (Johnson *et al.* 1949*c*), must not be forgotten. Our figures for rabbit red cells are similar to those reported by Kirk (1938) for man. It is interesting to note that most of the lipid of the red cell is attached to the cell stroma and that the lipid distribution in the insoluble material after the cells have been haemolysed is similar to that of the intact cells (Bürger & Beumer, 1913; Erickson *et al.* 1938; Erickson *et al.* 1940).

The results obtained for the polymorphonuclear leucocytes are in good agreement with those of Boyd & Stevenson (1937), who studied cholesterol, total phospholipin, total fatty acid and total lipid of rabbit white cells. Our figures for cholesterol are somewhat lower, but it should be remembered that Boyd & Stevenson (1937) were estimating the lipids of suspensions of the mixed white cells obtained by centrifuging rabbit blood. Boyd & Stephens (1936) suggested that lymphocytes may have a different lipid content from that of polymorphonuclear leucocytes. Also it is possible that the white cells studied by Boyd & Stevenson (1937) were contaminated with blood platelets, which are known to contain much lipid material (Erickson, Williams, Avrin & Lee, 1939). In addition to his results on the white cells of the rabbit, Boyd (1933, 1936*b*) obtained essentially similar figures for the lipids of mixed white cells obtained from the blood of man.

Boyd did not attempt to measure either cerebroside or the individual phospholipins of white cells. Our figures for the concentration of cerebroside in rabbit polymorphonuclear leucocytes were low and somewhat variable, but those for sphingomyelin were high and very consistent. We are not aware of any previous suggestion that white cells contain sphingomyelin. We have shown that the polymorphonuclear leucocytes of the rabbit contain a phospholipin that, on hydrolysis, behaves as a diaminophospholipin or phosphosphingoside. Limitation of material has precluded any attempt to isolate this substance from the white cells, but the isolation of sphingomyelin from the spleen (Thannhauser & Setz, 1936*a*; Thannhauser & Boncoddo, 1948) makes it probable that this phospholipin of the rabbit polymorphonuclear leucocytes is indeed sphingomyelin.

SUMMARY

1. The concentration of cerebroside, total and free cholesterol, total phospholipin, monoaminophospholipin, lecithin and total fatty acid has been determined in both the red cells and the polymorphonuclear leucocytes of the rabbit. From these figures has been calculated the concentration of ester cholesterol, sphingomyelin, kephalin, neutral fat and total lipid. The red cells were obtained from the peripheral blood and the polymorphonuclear leucocytes from sterile peritoneal exudates.

2. The polymorphonuclear leucocytes contained more total lipid than the red cells. This was true for every lipid constituent measured, but the greatest difference was in the concentration of neutral fat. The polymorphonuclear leucocytes contained over twelve times as much neutral fat as did the red cells.

3. The relative amount of the phospholipin in the two cell types was similar, but the distribution of the individual phospholipins was different. The red cells contained more kephalin and the polymorphonuclear leucocytes contained more sphingomyelin.

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Observations on a Growth Factor for *Leuconostoc citrovorum*

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Sauberlich & Baumann (1948) first reported the presence in liver extracts of a growth factor for *Leuconostoc citrovorum*. Such a factor has been found both in liver extracts and in concentrates of vitamin B_{12} prepared from the fermentation liquors of *Streptomyces griseus* (Lees & Emery, 1949). Our aim has been to produce a concentrate of the factor, free from vitamin B_{12}, in order that it might be tested clinically for activity against pernicious anaemia. To help achieve this, a microbiological tube assay was developed.

EXPERIMENTAL

The microbiological tube assay

The assay differs from the assay of vitamin B_{12} with *Lactobacillus leichmanii*, to be reported elsewhere (Lees, Tootill & Emery, 1950), only in the nature of the test organism and its maintenance and in the omission of tomato juice from the medium.

The test organism is strain ATCC 8081 of *L. citrovorum* (Sauberlich & Baumann, 1948), maintained by fortnightly transfer in stab culture in the medium: Difco yeast extract,

0·3; glucose, 0·5; agar, 1·5%. The assay inoculum is prepared from a 24 hr. culture in the basal medium used for *Lb. leichmanii* assay, omitting the tomato juice and incubating at 37°. The cells are centrifuged down, washed once in an equal vol. of physiological saline and diluted to opacity 4/8 of Burroughs-Wellcome (B.W.) Standards, and 0·5 ml. is used to inoculate each assay tube. The physical treatment of the assay tubes (i.e. medium volumes, autoclaving, etc.) is in every way identical with that used in the *Lb. leichmanii* assay.

A reference standard of purified liver extract was established. The extract was clinically active in the treatment of pernicious anaemia, containing 8 μg. vitamin B_{12}/ml. with a total solids of 45 mg./ml. This extract was assigned the potency of 50 L.C. (*L. citrovorum*) units/ml.

The organism responds to the factor between the doses of 1 and 0·06 L.C. units/assay tube. Experience has shown that reliable assays are obtained over the whole of the range, no interference by other factors having been experienced. No response is given by this organism to pure vitamin B_{12} at a level of 0·5 μg./assay tube or to a vitamin B_{12} concentrate at a level of 125 μg./assay tube.

Plate test

This was carried out with the single-strength basal-tube medium, solidified with 1·5% agar and inoculated with 4 ml. of inoculum of B.W. opacity 4 (as produced for the tube assay)/100 ml. medium. Medium (400 ml.) was poured on to a rectangular plate 43 × 35·5 cm. Developed paper-strip chromatograms were laid on the surface of the solidified medium and incubated overnight at 37°. Zones of growth were observed as described below and the R_F values were thereby obtained.

Purification of the factor

In view of the findings of Lyman & Prescott (1949*a*), it was considered that the use of acid-exchange resins should offer the simplest method of separating the two factors, the *L. citrovorum* factor being adsorbed on such resins, but not vitamin B_{12}.

A sample of the weak base anion-exchange resin polyvinylchloride-ethylenediamine was obtained from the Chemical Research Laboratory, Teddington. The resin (10 g.) was put in a glass column, 1 cm. in diameter. The resin was washed with 10% Na acetate buffer at pH 4·5 (250 ml.) and with water until the washings were acetate free. A liver extract (25 ml.) produced by the method of Laland & Klem (1936), containing the *L. citrovorum* factor at a concentration of 50 L.C. units/ml. and a potency of 1 L.C. unit/mg., was passed slowly through the column. The column was water washed. The filtrate contained 4% of the input factor, but over 80% of the input vitamin B_{12}. The column was eluted with the same acetate buffer (100 ml.) to give a dark orange eluate containing 40% of the original *L. citrovorum* activity. In order to determine its potency, attempts were made to extract the factor from this eluate with phenol, but yields were very poor. The final potency was only about 0·7 L.C. unit/mg., indicating that the material was still contaminated with acetic acid or Na acetate or both.

In order to avoid the use of acetate, the strong base anion exchange resin IRA 400 (Rohm and Haas Co.) was used for subsequent experiments. This resin was used in its OH^- form. Passage of a further portion of liver extract through a column of this resin resulted in 84% of the *L. citrovorum* activity being adsorbed. The column was eluted with 10% NaCl solution. The alkaline eluate contained 42% of the input *L. citrovorum* activity, but no *Lb. leichmanii* factor (vitamin B_{12}). Extraction of this eluate at pH 4 with phenol, butanol, amyl acetate and $CHCl_3$ gave such poor recoveries of activity that no salt-free potency could be determined. Vitamin B_{12} can be extracted from aqueous solution with $CHCl_3$ (Novak & Hauge, 1948), and thus differs from our factor.

A similar experiment was carried out with a vitamin B_{12} concentrate made from a *Strep. griseus* culture filtrate by an adaptation of the Laland & Klem (1936) process (i.e. charcoal adsorption and phenol elution). The IRA 400 column used was 21 cm. high and 2·5 cm. in diameter. The concentrate (100 ml.) containing 61 L.C. units/ml. (potency 0·85 unit/mg.), was passed slowly through the column. The filtrate contained 165 L.C. units (2·7%). The column was washed with water (500 ml.) and eluted with 10% (w/v) NaCl solution (400 ml.). The alkaline orange eluate contained 3800 L.C. units (62%) and was neutralized by passage through a column (15 cm. high, 2·5 cm. diameter) of the base-exchange resin IRC 50 (Rohm and Haas Co.) in its H^+ form. The filtrate, pH 3, was completely inactive and almost colourless. The column was washed with water to give a yellow eluate containing 2900 L.C. units (47·3%), with a potency of 8·8 L.C. units/mg.

To confirm this experiment, 900 ml. of bulked IRA 400 eluates from several experiments, containing 10,800 L.C. units, were brought to pH 11 and passed through a similar column of IRC 50. The filtrate contained 220 L.C. units (2%). The column was eluted with water. The first 75 ml. of eluate contained no activity, the next 200 ml. contained 9000 L.C. units (83%), with a potency of 7·8 L.C. units/mg., and a further 300 ml. the remaining 2000 units at a lower potency.

A sample of the main eluate, concentrated to 100 L.C. units/ml., was tested clinically for anti-pernicious anaemia activity at a 3 ml. level.

RESULTS

Experiments have been carried out to compare the stabilities of the *L. citrovorum* factor and vitamin B_{12}. A commercial purified liver extract and a concentrate prepared from *Strep. griseus* fermentation liquors (S.B. 8) were autoclaved (45 min. at 15 lb.). The concentrate from *Strep. griseus* fermentation liquors was stored at 37° after adjustment to various pH's. Pure crystalline vitamin B_{12} was autoclaved (45 min. at 15 lb.) and its degradation products were tested against both *Lb. leichmanii* and *L. citrovorum*. Results are shown in Table 1.

These experiments show that (1) the *L. citrovorum* factor is more stable to autoclaving than the *Lb. leichmanii* factor. (2) The *L. citrovorum* factor is least stable at acid pH, whilst the *Lb. leichmanii* factor is least stable at alkaline pH, indicating that both factors are least stable in the free ionized forms, since Lyman & Prestcott (1949*a*) refer to the separation of factors stimulating these two organisms by electrolysis as evidence that the *L. citrovorum* factor

contains a strong acidic group and the *Lb. leichmanii* factor a basic group. (3) The degradation products of vitamin B_{12} do not stimulate *L. citrovorum*.

Table 1. *Activities of preparations of* Lb. leichmanii *and* Leuconostoc citrovorum *factors*

Sample	*Leuconostoc* assay (L.C. units/ml.)	*Lactobacillus* 313 assay (μg./ml.)
Liver extract/263H	50	8·0
Liver extract/263H (autoclaved 45 min. at 15 lb.)	44	3·9
S.B. 8	11·5	8·0
S.B. 8 (autoclaved)	6·0	0·7
S.B. 8 stored 13 days at 37°:		
pH 4	<2·0	5·5
pH 6	7·5	3·5
pH 8	11·5	1·0
pH 10	11·5	0·2
B_{12} original solution	<0·2	1·0
B_{12} autoclaved	<0·2	0·1

DISCUSSION

The fact that *L. citrovorum* factor is adsorbed on anion-exchange resins confirms its acidic nature. The adsorption of activity from alkaline solution on the cation-exchange resin IRC 50 and its subsequent elution with water indicate the presence of a weak basic group in the molecule. It was noted that the riboflavin present in the original concentrates, whether prepared from liver or from culture filtrates, was isolated in the same fractions as the *L. citrovorum* factor. This observation is of interest, as riboflavin is itself amphoteric.

A number of samples containing the factor were examined by the method of paper-strip chromatography. The developing solvent was butanol-acetic acid (9:1) saturated with water. After development, the strips were dried and examined on plates inoculated with *L. citrovorum*. Under the conditions used, the *L. citrovorum* factor was found to have an R_F value of about 0·7, compared with an R_F value of about 0·3 for vitamin B_{12} (on *Lb. leichmanii* plates) and 0·8 for thymidine (with both organisms).

Clinical testing of a concentrate of the factor, free from vitamin B_{12}, in pernicious anaemia, showed that it gave no erythropoietic response. A recent paper by Laland & Dedichen (1949) indicated the presence of leucocyte-stimulating activity in purified liver extracts. In a personal communication, Dr Jens Dedichen has told us that a concentrate of our citrovorum factor showed similar activity. This will be further investigated and reported on in due course. After this work had been completed, a paper by Lyman & Prescott (1949*b*) appeared, stating that the citrovorum factor was retained on IRA 400, while *Lb. leichmanii* activity was not. They also mentioned the stability of the factor to alkali. Further, Broquist, Stokstad, Hoffmann, Belt & Jukes (1949) have published 'Some observations on growth factors required by *Leuconostoc citrovorum*'. Their factor, separated by paper-strip chromatography, may well be identical with the one discussed above. The activity of their fraction was also stable to alkali.

SUMMARY

1. Liver extracts and fermentation liquors from *Streptomyces griseus* contain a growth factor for *Leuconostoc citrovorum*. A microbiological tube test has been developed for this factor, and an arbitrary unit has been defined.

2. The factor is not identical with vitamin B_{12} or vitamin B_{13}.

3. Concentrates of the factor derived from liver or from fermentation liquors, and containing vitamin B_{12} also, have been purified approximately ten times, and the vitamin B_{12} has been removed, by adsorption on the anion-exchange resin IRA 400, elution with sodium chloride solution, adsorption of the alkaline eluate on the cation-exchange resin IRC 50 and elution with water.

4. The factor appears to have no activity against pernicious anaemia, but possesses leucocyte-stimulating activity.

We wish to acknowledge the assistance of Miss A. Gatiss and Mr D. Goodinson in purification and assay work.

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Biochemical and Clinical Changes in the Rabbit Lens during Alloxan Diabetes

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The nature of the chemical changes underlying the formation of lens opacities is still not clear. Much work has been done on human cataractous lenses, but analysis of these can only describe the end result of processes leading to cataract, because no lens is removed from a human eye until opacity is complete. Production of experimental cataract in animals has made possible the study of the chemistry and metabolism of the lens in the pre- and early cataractous stages, and such studies may show what chemical changes precede or accompany opacity formation. Bellows (1944) gives a summary of the work already done in this field. It has been established (e.g. Reis, 1912; Shoji, 1927, 1931; Cohen, Kamner & Killian, 1927; Gifford, 1932; Bellows & Rosner, 1937; Rosner, Farmer & Bellows, 1938) that the fully cataractous lens gives a much reduced reaction with sodium nitroprusside. This has been attributed to a decrease in glutathione content which in the normal rabbit lens averages 320 mg./100 g. (Bellows, 1944), equivalent to 34·6 mg. sulphur/100 g. The normal eye also contains a high concentration of ascorbic acid within the fluids and tissues. The high concentration of glutathione and ascorbic acid make it possible that these substances are concerned with the metabolism of the lens, and it seemed interesting to determine whether they are reduced in amount early in cataract formation or whether there is no change until the opacity is well advanced.

Cataracts produced in rabbits made diabetic with alloxan were used as experimental material. Dunn, Sheehan & McLetchie (1943), following the earlier work of Jacobs (1937), found that intravenous injection of alloxan produced permanent diabetes in animals. The pathology of alloxan diabetes has been fully discussed by Bell (1948) and by Lukens (1948). Bailey, Bailey & Leach (1944) first reported cataract in such animals and this was confirmed by Duffy (1945). Both authors found that cataract did not occur in every diabetic animal. This is borne out in my experiments. The cataract which occurs in alloxan diabetes closely resembles the type of cataract seen in young human diabetics. It therefore seemed reasonable to use alloxan-induced cataract in spite of the uncertainty with which it appears in diabetic rabbits.

METHODS

Induction of diabetes. Normal young adult rabbits, mostly small Dutch, were given intravenous injections of alloxan in doses of 175–200 mg./kg. body weight. The alloxan, dissolved in about 10 ml. sterile water, was injected slowly into the marginal ear vein in a single dose spread over a period of 5 min. or more. Since the injection appeared painful the vein in the neighbourhood of the injection site was anaesthetized by subcutaneous injection of procaine hydrochloride 5% (w/v) in liq. adrenal. 1/100,000. Each animal was closely watched during the 60 hr. following injection. The animals usually developed signs of hypoglycaemia 4–10 hr. after injection of alloxan, when 50% (w/v) glucose solution was administered, if necessary intravenously, but usually by stomach tube, to prevent convulsions. A high blood sugar usually developed 24–48 hr. later, although there was considerable variation. The hyperglycaemia was controlled for the first few days by intramuscular injection of insulin (20 units/ml.); 1–4 units/day were generally required. Occasionally a rabbit developed lipaemia. This was noticed by a 'cream' appearing on the surface of blood taken for sugar estimation; attempts were made to control it by giving balanced injections of glucose and insulin, but in only one case could the lipaemia be cured; most of these animals died within 3 or 4 days. All animals were examined often during the experiment and the blood-sugar level closely followed. The eye condition was studied by slit-lamp examination and by ophthalmoscopy after dilatation of the pupil with atropine. In three animals iridectomy was performed in order to see the periphery of the lens more clearly.

Blood sugar. The sugar content of blood taken from the marginal ear vein was determined by the ferricyanide method (Kramer & Steiner, 1931; Hagedorn & Jensen, 1923).

Ascorbic acid. Ascorbic acid in the aqueous humour and lens was determined by titration with 2:6-dichlorophenol-indophenol, using the method of Bessey & King (1933). Aqueous humour was removed with a fine capillary pipette immediately after death, but before the eye was removed, and transferred to a centrifuge tube containing 0·2 ml. of 30% metaphosphoric acid. The volume was made up to 3 ml. with distilled water. Any precipitate was centrifuged off and the ascorbic acid in the supernatant liquid titrated against the dye. The lens, without capsule, was removed from the eye, weighed, dropped into a mortar containing 1 ml. 30% metaphosphoric acid and ground to a paste; 1 ml. distilled water was added and grinding continued until the mixture was homogeneous. The suspension was washed into a centrifuge tube with two 1 ml. portions of

distilled water. After centrifuging, the ascorbic acid in the supernatant fluid was titrated.

Thiol sulphur. Thiol S is present in the lens as the —SH groups of glutathione and of the lens proteins. The —SH content was determined in both fractions.

Glutathione. The glutathione —SH in a portion of the supernatant fluid used for the ascorbic acid estimation was determined by titration against 0·005 N-I_2 in KI solution by the method of Perlzweig & Delrue (1927). The I_2 value of the ascorbic acid present was calculated from the dye titration and subtracted from the total I_2 titration figure.

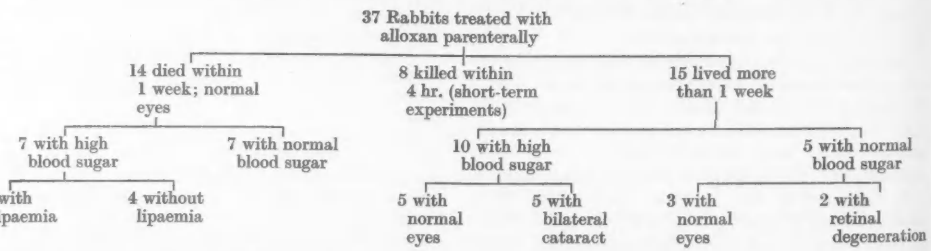
Total —SH. Chloroacetophenone (CAP) was used to estimate the —SH groups in whole lens solution, as described by Dixon (1948). Whole lens, without capsule, was weighed, ground with water to form an emulsion and made up to 5 ml. The —SH content of this solution was determined by reaction with CAP in Warburg manometers. To each cup was added 0·5 ml. of 1·3% (w/v) $NaHCO_3$ solution and 1·5 ml. water and, in the side arm, 0·02 ml. of 10% (w/v) solution of CAP in ethylene glycol monoethyl ether. In addition, 1 ml. of lens solution was added to the liquid in the body of the flask and the apparatus filled with N_2 containing 5% (v/v) CO_2 in the usual way. After equilibration the taps were closed and the reagents mixed. One mol. CO_2 is liberated for each g.equiv. of —SH present. The manometers were read after 10 min., by which time the reaction was complete. Controls containing lens solution alone and CAP alone were run at the same time.

Alloxan. This was determined using the method described by Archibald (1945) in which the alloxan is coupled with *o*-phenylene diamine and the fluorescence of the resulting compound measured.

RESULTS

Clinical. The general pattern of the results is summarized in Table 1. In the short-term experiments the animals were killed within 4 hr. of the completion of the alloxan injection. No clinical change in the eyes was seen during this time.

Table 1. *Summary of general results of alloxan treatment*


37 Rabbits treated with alloxan parenterally
14 died within 1 week; normal eyes
8 killed within 4 hr. (short-term experiments)
15 lived more than 1 week
7 with high blood sugar
7 with normal blood sugar
10 with high blood sugar
5 with normal blood sugar
3 with lipaemia
4 without lipaemia
5 with normal eyes
5 with bilateral cataract
3 with normal eyes
2 with retinal degeneration

Five of the surviving rabbits with blood-sugar values exceeding 300 mg. glucose/100 ml. developed bilateral posterior polar cataract 4–6 weeks after alloxan treatment. The first sign appeared as a slight 'furring' of the suture lines on the posterior pole of the lens. This opacity gradually spread in two definite stages. The first, more rapidly moving veil had the appearance of dust on a glass plate. It could be seen quite clearly by reflected light and occasionally rather indistinct radial spokes could be seen. The second stage was a more slowly developing opacity which had a chalky appearance. This dense white opacity could be seen with the unaided eye. In three animals vacuoles and irregular water clefts could be seen, especially where an iridectomy had been performed. The opacities were always bilateral, were only seen at the posterior pole, and never covered more than one-half the area of the lens. There was some discontinuity of refractive index below the anterior surface of the lens, although this did not amount to an opacity.

Two animals with normal blood-sugar values developed retinal degeneration.

A general pathological examination was made of one animal in which the blood-sugar value exceeded 700 mg./100 ml. for 6 days before death. This confirmed the fact that the main pathological change is in the pancreas where characteristic degeneration of the β-cells was found with no change in the acinar tissue.

Estimation of ascorbic acid and glutathione. The results of analysis of the aqueous humour and lens are set out in Tables 2 and 3. Table 2 shows that the ascorbic acid content of the aqueous humour and of the lens did not change during the experiment. The values show a wide scatter. Table 3 summarizes the analysis of the glutathione and protein thiol groups. The glutathione content of the lens after injection of alloxan is very variable, but in no case differs significantly from normal. On the other hand, the total —SH content of the lens decreased immediately after alloxan injection, and did not regain the normal value during the whole period of the experiment. Some rabbits lived 2 years after alloxan injection and the —SH content of the lens was still low when they were killed. Since the glutathione content appeared to remain normal this decrease in —SH must be due to a decrease in the thiol groups of the lens proteins. The last column of Table 3 gives the values for 'protein —SH' obtained by subtracting the mean value for the total —SH.

Table 2. *Ascorbic acid content of normal and alloxan-treated rabbit lens and aqueous humour*

(Figures given in order are: Mean ± S.D. (extreme values), number of observations.)

	Aqueous humour (mg./100 ml.)	Lens (mg./100 ml.)
Normal	34·3 ± 3·6 (28·6–40·6) 8	12·1 ± 4·2 (6·9–19·0) 9
<4 hr. after alloxan; short-term experiments	36·2 ± 10 (23·5–50) 7	11·2 ± 3·3 (6·9–18·5) 11
>2 weeks after alloxan; high blood sugar	37·7 ± 10 (22–54) 5	9·0 ± 2·6 (5·3–10·8) 5
Bilateral cataract; high blood sugar	36·7 ± 13 (22–54·5) 5	8·1 ± 2·2 (5·3–10·7) 3

Table 3. *Thiol content of the lens of rabbits treated with alloxan*

(Figures given in order are: Mean ± S.D. (extreme values), number of observations. Values for 'protein —SH' obtained by subtracting glutathione —SH from total —SH.)

	Glutathione (iodine) (mg. S/100 g. lens)	Total —SH (CAP) (mg. S/100 g. lens)	'Protein —SH' (mg. S/100 g. lens)
Normal	34·0 ± 7 (27–43) 3	187 ± 9·2 (173–195) 6	153
<4 hr. after alloxan; short-term experiments	55 ± 20 (19–95) 11	85 ± 19 (62–110) 8	30
>2 weeks after alloxan; normal blood sugar	35 ± 3 (30–43) 3	115 ± 9 (108–128) 3	80
>2 weeks after alloxan; high blood sugar	34·0 ± 7 (29–40) 5	101 ± 10 (93–115) 5	67
Bilateral cataract; high blood sugar	28 ± 3 (25–32) 3	108 ± 7·4 (98–115) 3	80

Effect of precipitant. Pirie (1946) found that oxidized glutathione present in solution with dialysed protein became reduced when treated with certain precipitating agents such as trichloroacetic acid. Metaphosphoric acid, however, seemed to be free from this effect. I have confirmed this using cystine. Lens solution dialysed to remove glutathione was mixed with a small quantity of cystine and the protein precipitated with 30% (w/v) metaphosphoric acid. The supernatant fluid after centrifuging gave a negative nitroprusside reaction. Application of the CAP method to this solution was made difficult by the buffering action of the phosphate. Removal as the barium salt gave an unbuffered solution which appeared not to react with CAP. The effect of precipitating agents on the state of glutathione is being investigated further.

Fate of alloxan. Alloxan disappeared from the blood and from the aqueous humour during the inevitable but short interval between the completion of the injection and removal of the fluids. Less than 2 μg. was found in 0·2 ml. samples of either fluid, whereas, had the alloxan distributed itself evenly throughout the body and not reacted with the tissues, the samples would have contained at least 25–30 μg.

DISCUSSION

Two main oxidation-reduction systems involving thiol groups are thought to be present in the lens. Bellows (1944) suggests that hydrogen from a substrate is transferred to the sulphur of the protein; this in turn transfers hydrogen to glutathione and thence to dehydroascorbic acid. The reactions may be represented as follows:

(1) $RH_2 + PSSP \rightleftharpoons 2PSH + R$,

(2) $GSSG + 2PSH \rightleftharpoons PSSP + 2GSH$,

(3) 2GSH + dehydroascorbic acid ⇌ GSSG + ascorbic acid,

where RH_2 = substrate, P = protein residue, G = glutathione residue.

If we consider these suggested processes in the reverse order, reaction (3) is known to proceed. Hopkins & Crook (1938) have shown that ascorbic acid is protected from oxidation in the presence of glutathione which is itself oxidized. Reaction (2) also occurs. Hopkins & Morgan (1938) showed that the thiol groups in succinic dehydrogenase can be oxidized by glutathione and that this inactivates the enzyme. Lens proteins can also reduce glutathione in this way. Reaction (1) has not been demonstrated. The proposed scheme is therefore unproved, but perhaps remains a possible path of lens metabolism.

The results given here suggest that alloxan combines with some of the thiol groups in the lens. Alloxan may have a selective affinity for the thiol groups of the protein, but this seems unlikely; it is more probable that alloxan combines with protein and glutathione in equivalent amounts. If reaction (2) is more rapid than reaction (1), reduced protein (PSH) will be re-oxidized by glutathione (GSSG) as

soon as it is formed. Combination of glutathione with lens protein as suggested by Hermann & Moses (1945) would also explain the maintenance of glutatione concentration at the expense of the protein —SH. A drop in thiol content, or, more accurately, a decrease in the intensity of the nitroprusside reaction has been reported many times, both in clinical and experimental cataract. This change has been attributed to a change in the glutathione concentration, but in view of the results given here it is suggested that this is secondary to a change in the protein of the lens.

This change in reducing sulphur is not, in itself, responsible for the formation of cataract as it occurred in animals that did not show lens changes. All the rabbits that developed cataract had a high blood-sugar level, but out of ten with high blood-sugar levels only five developed cataract. At present no known change either in the chemistry of the lens or in the general metabolism of alloxan-treated rabbits can be shown to lead in all cases to cataract formation. The results reported here showing that an immediate change occurs in the lens proteins after injection of alloxan make it probable that other early changes also take place. Study of these may be expected to increase our knowledge of cataract formation more than studies on the cataractous lens itself.

SUMMARY

1. Clinical and biochemical changes occurring in the eyes of rabbits during alloxan diabetes are described.

2. An attempt is made to explain the reduction in the amount of protein —SH on the basis of the suggested sulphur-containing oxidation-reduction systems of the lens.

I wish to acknowledge with gratitude my indebtedness to Miss Ida Mann for her advice and encouragement during the early part of this work and for performing the iridectomy operations, and to Dr W. E. Gye for examining the histological specimens. I also wish to thank Dr A. Pirie for her continued guidance and helpful criticism throughout the whole of these investigations.

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The Micro-estimation and Origin of Trimethylamine in *Chenopodium vulvaria* L.

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The presence of trimethylamine in the tissues of *Chenopodium vulvaria* L. was first established by Dessaignes (1852). Further observations on the occurrence of this base in *Ch. vulvaria* were made by Wicke (1862) and Klein & Steiner (1928). Trimethylamine is present in small amounts in the flowers of many plants and occurs with methylamine in the rhizome of *Acorus Calamus* and in the young shoots of *Mercurialis annua* (Guggenheim, 1940). Challinor (1913) has reported the occurrence of trimethylamine in the tissues of *Rhagodia hastata*, and Kapeller-Adler & Vering (1931) showed that the concentration of trimethylamine may reach the value of 500 mg./100 g. dry weight in the tissues of the red algae *Ceramium rubrum* and *Rhodomela subfusca*. Trimethylamine diffuses from innumerable glands

on the surface of the stems, leaves and perianth of *Ch. vulvaria*, thus distinguishing this species from most, if not all others, of the genus. The immediate precursor of trimethylamine in *Ch. vulvaria* may be either choline or betaine as both these substances are present in the free state in the tissues. Steele (1949) states that in *Ch. vulvaria* trimethylamine is a decomposition product of betaine, but experimental evidence in support of this statement appears to be lacking. The object of the present work was to determine the mode of formation of trimethylamine in this plant and to study the enzyme system involved. For the micro-estimation of trimethylamine in fish muscle, Dyer (1945) used a modification of the picrate method used by Richter, Lee & Hill (1941) for the determination of amines in blood. However, neither this method nor the titrimetric method gave entirely satisfactory results when applied to the determination of trimethylamine in the tissues of *Ch. vulvaria*, and it became necessary to develop a more specific micromethod for the estimation of this amine. Palumbo (1948) observed that tertiary amines in the presence of *cis*-aconitic anhydride dissolved in acetic anhydride give reddish purple colours, while ammonia and primary and secondary amines do not react. This reaction was studied and adapted for the colorimetric estimation of trimethylamine in *Ch. vulvaria*.

EXPERIMENTAL AND RESULTS

Reagents

Cis-*aconitic anhydride*. Prepared from aconitic acid by the method of Malachowski & Maslowski (1928). The product was purified by recrystallizing four times from hot benzene. A 2·5 % solution in glacial acetic acid was freshly prepared before use.

Glacial acetic acid, A.R.; *acetic anhydride*, A.R.; *toluene*, sulphur free; *choline chloride*; *betaine hydrochloride*; Na_2SO_4 (anhydrous), A.R.; K_2CO_3, A.R.

The micro-estimation of trimethylamine

Procedure. A solution (4 or 5 ml.) containing not more than 100 μg. of trimethylamine was run into a 25 ml. cylindrical separating funnel followed by 10 ml. of toluene. A saturated solution of K_2CO_3 (3 ml.) was added, the funnel shaken vigorously for 1 min. and allowed to stand for 5 min. The K_2CO_3 solution was run off and the toluene solution of trimethylamine poured into a test tube containing 0·5 g. anhydrous Na_2SO_4. The tube was corked and agitated for 10 sec. Into a second tube 0·1 ml. of the *cis*-aconitic anhydride reagent, 2 ml. of acetic anhydride and 5 ml. of the toluene solution were pipetted in this order. The solutions were thoroughly mixed and the tube, lightly corked, was placed in a constant-temperature water bath at 65° for exactly 10 min. The contents of the tube were cooled to room temperature and after an interval of 15 min. the colour was read in the Spekker absorptiometer using the Ilford spectrum yellow-green filter. A standard solution of pure trimethylamine hydrochloride was used for the calibration curve. Fig. 1 shows absorptiometer readings (extinctions, *E*) plotted against concentrations in μg. of trimethylamine.

Comments on the method

A solution of trimethylamine in toluene gives, with the *cis*-aconitic anhydride reagent and acetic anyhdride at 65°, a claret colour the intensity of which is proportional to the concentration of trimethylamine between the limits of 5–50 μg./5 ml. of toluene solution. The colour is stable for at least 30 min. after development, but fades gradually on long

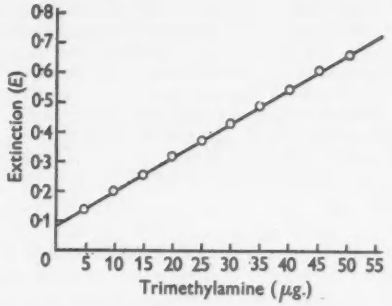

Fig. 1. Calibration curve obtained with a standard solution of trimethylamine hydrochloride. (Drum of absorptiometer set to 1 with toluene blank. 0·5 cm. cells.)

standing. Other tertiary amines give red or purple colours with the reagent and, if not removed, interfere in the determination of trimethylamine. It has been found, however, that distillation at low temperature under reduced pressure removes trimethylamine from most of the tertiary amines likely to interfere. Toluene of the grade specified gives a faint pink colour with the reagent and it is necessary, therefore, to run a control blank with each set of estimations. It is important to observe the details of time and temperature in the development of the colour and for each fresh batch of reagents a calibration curve is essential. The solution of trimethylamine in toluene should be added as soon as possible to the mixture of the *cis*-aconitic anhydride reagent and acetic anhydride, otherwise a red colour gradually develops in the mixture on long standing at room temperature. Immediate addition of the toluene solution prevents the development of the colour.

Application of the method to the determination of trimethylamine in Chenopodium vulvaria *L.*

Accurate estimation of trimethylamine in *Ch. vulvaria* is rendered difficult by the fact that this volatile base diffuses freely from the epidermal glands. These glands are very delicate and are easily ruptured on handling. The factors controlling the liberation of trimethylamine from the glands have not yet been fully determined, but it has been observed that atmospheric humidity and temperature are of importance in this connexion. Furthermore, the pH value of the epidermal cells and leaf tissues was found to be 6·5, and that of the glands 8·2. Variation in the pH value of the glands may occur, however, and thus influence the rate of diffusion of trimethylamine from the glands.

Drying of the tissue results in considerable loss of trimethylamine, therefore it is essential to use fresh tissues for analysis. A preliminary examination of the trimethylamine content of whole leaves, and leaves from which the glands had been carefully removed with a soft brush, was made as follows. Leaves (0·5 g.) were detached with forceps from individual plants (raised from seed) at two stages of growth, i.e. young plants showing active vegetative growth and fully mature plants in flower. The leaves were ground to a paste in a mortar with 1 ml. N-HCl, 10 ml. distilled water were added and the extract transferred to the vacuum distillation apparatus. Antifoam solution (10 g. paraffin, m.p. 54°, in 100 ml. toluene) (1 ml.) and excess $Mg(OH)_2$ were added and the mixture distilled under reduced pressure at 40° into standard acid (5 ml.) until the total volume of the distillate reached 20 ml. The rate of distillation was controlled to give 15 ml. of distillate in 30 min. The distillate was made up to 25 or 50 ml. and a sample of 4 ml. taken for analysis.

Table 1. *Trimethylamine content of leaves of* Chenopodium vulvaria *L.*

	Trimethylamine (μg./0·5 g. wet wt.)					
	Young plants			Plants in flower		
Plant no.	Intact leaves	Leaves without glands	Glands	Intact leaves	Leaves without glands	Glands
1	850	265	585	425	125	300
2	818	221	597	470	137	333
3	740	218	522	580	145	435
4	890	282	608	326	102	224
5	730	248	482	410	116	294
6	805	260	545	370	110	260
Mean	**805**	**249**	**556**	**430**	**122**	**308**

Table 1 shows the values for trimethylamine in intact leaves, leaves from which the glands had been removed, and (by difference) the glands.

The origin of trimethylamine in Chenopodium vulvaria *L.*

Enzymic breakdown of choline by certain members of the Enterobacteriaceae has been reported by Dyer & Wood (1947). These workers showed that trimethylamine is produced at pH 7·2 from choline and acetylcholine, but not from betaine. Cohen, Nisman & Raynaud (1947) found that certain species of *Clostridium* break down choline with the production of trimethylamine, and ethanolamine with the production of ammonia and ethylene glycol.

Preparation of an active enzyme extract from leaves of Chenopodium vulvaria *L.* The leaves (50–100 g.) were frozen with solid CO_2, allowed to thaw and pressed in a powerful hydraulic press. The press cake was allowed to imbibe 100 ml. of 5% (v/v) glycerol solution and again pressed out. The press juice and glycerol extract were combined (130 ml.) and dialysed against distilled water at 2° for 18 hr. At the completion of dialysis the enzyme extract contained small amounts only of choline, betaine and trimethylamine. The enzyme preparation (2 ml.) was placed in each of six 25 ml. conical flasks (*a–f*) and 3 ml. 0·1 M-phosphate buffer of the required pH were added. Flasks *a–d* each received 1 ml. of a 0·01 M-solution of choline chloride or betaine hydrochloride (previously neutralized to pH 7). Before the addition of buffer and substrate, the enzyme solution in flasks *c* and *d* was heated to 100° and cooled. Flasks *e* and *f* were used as controls. A crystal of thymol was added to each flask and the solutions allowed to stand at 24° for 48 hr. After this period the contents of each flask were transferred to the distillation apparatus, 1 ml. antifoam solution and excess of $Mg(OH)_2$ added, and distillation carried out for 20 min. under reduced pressure at 40° into 5 ml. standard acid. The distillate was made up to 25 ml. and 4 ml. were taken for the determination of trimethylamine.

The results of this experiment, which are set out in Table 2, show that trimethylamine is produced by the enzyme preparation at pH 7·8, but not at pH 6 from choline. Apparently no trimethylamine is formed from betaine. The specificity of action of the enzyme was examined by substitution of ethanolamine, *N*-methylethanolamine and dimethylethanolamine for choline in the above experiment. The enzyme was without action on these amines. An extract prepared in the same manner from leaves of *Ch. album* L. was used in a parallel experiment. Trimethylamine did not appear as a breakdown

Table 2. *Production of trimethylamine from choline and betaine by an enzyme preparation from leaves of* Chenopodium vulvaria *L.*

(Flasks *a–d* contained either choline or betaine, enzyme solution and buffer; flasks *e* and *f* contained enzyme solution and buffer only; flasks *c* and *d* were heated to 100° and cooled.)

Flask ...	*a*	*b*	*c*	*d*	*e*	*f*
pH ...	7·8	6·0	7·8	6·0	7·8	6·0
	Trimethylamine (μg./2 ml. distillate mean value of 3 experiments)					
Choline	53	9	12	9	6	6
Betaine	8	7	8	8	6	6

product of either choline or betaine. Glycollic acid could not be detected as a product of the action of the enzyme on choline, but the presence of ethylene glycol was established by periodate oxidation to formaldehyde which was detected by the Schiff reaction (Feigl, 1946). It was not possible to isolate a derivative of this alcohol. The detection of formaldehyde after periodate oxidation indicates that the enzyme is a choline aminase similar to that found by Cohen *et al.* (1947) in bacteria. Attempts to purify the enzyme by precipitation with acetone and ammonium sulphate resulted in complete inactivation.

DISCUSSION

In a previous publication (Cromwell, 1949), a scheme showing the possible reactions leading to the formation of methylated amines in plants was formulated, and it was suggested that trimethylamine is derived from choline. The results of the present work show that in *Ch. vulvaria* enzymic breakdown of choline takes place with the production of trimethylamine. Betaine, which has also been considered to undergo breakdown with the formation of trimethylamine, is not attacked. Preliminary examination of the leaves of *Ch. vulvaria* at different stages of development of the plant shows that production of trimethylamine is greatest during the phase of active vegetative growth. As the plant reaches maturity the production of trimethylamine declines. Ducet (1948) has shown that during vegetative growth of the soya bean there is a tendency for synthesis of phospholipins to take place at the expense of soluble choline (free choline plus glycerophosphoryl choline), whereas during maturation of the seed, degradation of phospholipins takes place with the liberation of choline. If, as in Soya, the content of free choline in *Ch. vulvaria* is diminished during vegetative growth by synthesis of phospholipin it would be expected that as the plant reaches maturity the amount of choline available for the production of trimethylamine would show a progressive decline. The limited scope of the present experiments, however, makes it impossible to decide whether free choline is attacked by the enzyme before synthesis of phospholipins takes place, or whether degradation of phospholipins yields the choline, which is subsequently broken down to trimethylamine. Hanahan & Chaikoff (1947, 1948) have shown that an enzyme capable of degrading phospholipins with the production of choline is present in the leaves of the carrot and cabbage. If choline acts as a methyl donor in plants its capacity to do so in *Ch. vulvaria* must be restricted by its tendency to lose all three methyl groups in combination with nitrogen as trimethylamine. On the other hand, betaine, which does not undergo breakdown to trimethylamine, may act as the donor of one methyl group in the process of transmethylation. In the other species of *Chenopodium* examined, trimethylamine does not occur as a breakdown product of choline. However, it is interesting to note that glands similar to those of *Ch. vulvaria* are present on the leaves of *Ch. album* and *Ch. glaucum*. Dyer & Wood (1947) found that, in the absence of available carbohydrate, organic acid was produced in the bacterial metabolism of choline. These workers suggest that the alcoholic side chain of choline is oxidized serving as a source of energy and trimethylamine is a by-product of the reaction. Ehrlich & Lange (1913) have shown that the yeast *Willia anomala* Hansen (= *Hansenula anomala* Hansen Syd.) is capable of growth when supplied with betaine as the sole source of nitrogen. Glycollic acid was produced in the culture medium, but trimethylamine was not detected. It is presumed that either trimethylamine was produced and immediately metabolized with the production of ammonia, or degradation of betaine took place by way of dimethylglycine with ultimate production of ammonia. In the experiments of Ehrlich & Lange (1913) and of Dyer & Wood (1947) the organisms were cultivated under abnormal conditions, the media lacking carbohydrate or a normal source of nitrogen. In *Ch. vulvaria* trimethylamine production, with consequent loss to the plant of nitrogen, is a feature of normal metabolism. Further work is in progress which, it is hoped, will throw more light on the nitrogen metabolism of this remarkable plant.

SUMMARY

1. A sensitive method for the micro-estimation of trimethylamine has been developed and employed for the estimation of trimethylamine in the leaves of *Chenopodium vulvaria* L.

2. An enzyme preparation from the leaves of *Ch. vulvaria* L. has been shown to cause breakdown, at pH 7·8, of added choline with the liberation of trimethylamine.

3. Betaine does not yield trimethylamine when added to this enzyme preparation.

4. The enzyme is inactivated by precipitation with ammonium sulphate and acetone and is sensitive to heat and changes of pH.

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The Synthesis of Peptides Related to Gramicidin S and the Significance of Optical Configuration in Antibiotic Peptides

2. PENTAPEPTIDES

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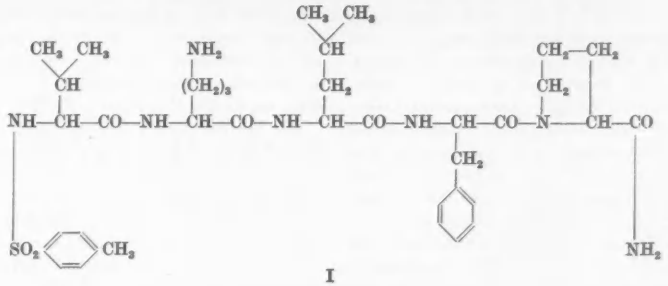
In Part 1 (Harris & Work, 1950) we reported that esters of both the tripeptides L-leucyl-L-phenylalanyl-L-proline and L-leucyl-D-phenylalanyl-L-proline synthesized by us showed some antibacterial action, but that they were very much less active than the natural antibiotic, gramicidin S, upon which they were modelled. Since there was no clear-cut distinction between the activities of the two optically isomeric peptides, it was decided to extend the peptide chain and to synthesize a pentapeptide containing all the amino-acid constituents of gramicidin S in their proper sequence and optical form, L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl-(II). For comparison with this peptide we synthesized the optically isomeric pentapeptide containing L-phenylalanine in place of D-phenylalanine. By comparison of the antibacterial activity of these two peptides we hoped to reveal the part played by D-phenylalanine in the production of antibiotic action.

Our two isomeric open-chain pentapeptides, although comparable one with the other, are not quite comparable with gramicidin S since they possess, in addition to the free δ-amino group of the ornithine residue, a free carboxyl and a free α-amino group, both of which are absent from the natural antibiotic. The absence of reactive end groups in gramicidin S is most satisfactorily explained by the assumption of a cyclic structure. We scarcely hoped to achieve controlled cyclization of so complex a molecule as our synthetic 'D' pentapetide and therefore approached the problem by synthesis of an acylpentapeptide amide, *α-(p-toluenesulphonyl-L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline-amide*. This compound (I) was comparable to gramicidin S (II) in that it possessed the correct amino-acid sequence, the correct optical form and the desired absence of reactive end groups.

For comparison with the acylpentapeptide possessing a free δ-NH_2 on the ornithine residue, we intended at first to prepare the corresponding pentapeptide with the α-NH_2 of valine free and the δ-NH_2 of ornithine acylated, but, as explained below, this last project was abandoned in view of unexpected experimental difficulties and because of the limited biological activity of our first acylpentapeptide amide.

The choice of the sequence valylornithylleucylphenylalanylprolyl was dictated by experimental rather than theoretical considerations. Since gramicidin S is believed to be cyclic, any one of the five constituent amino-acids can be terminal in an open-chain analogue. Preliminary experiments indicated that some of the possible intermediates would be easier to synthesize and purify than others, and the sequence finally chosen was that which seemed to



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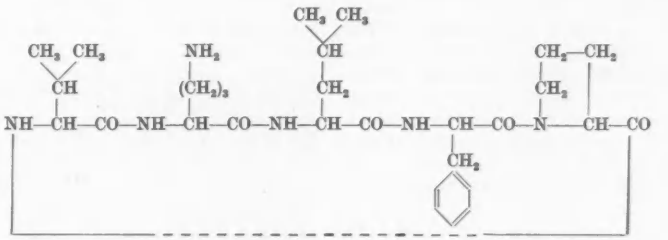

II

offer the best chance of complete purification and characterization of the intermediate and final products.

Synthetic procedures

The tripeptide esters, L-leucyl-D-phenylalanyl-L-proline methyl ester and L-leucyl-L-phenylalanyl-L-proline ethyl ester were already available from an earlier investigation (Harris & Work, 1950). For preliminary experiments on the lengthening of the peptide chain we used the readily obtainable DL-ornithine rather than the less accessible L-ornithine. *δ-Carbobenzyloxy-DL-ornithine* was prepared initially by the method developed by Neuberger & Sanger (1943) for δ-carbobenzyloxylysine. The yield (49 %) was disappointing. *αδ-Dicarbobenzyloxy-DL-ornithine* was obtained as a by-product. After the completion of these experiments, Synge (1948) also reported a low yield (40 %). In later experiments, using L-ornithine, we found that an excess of alkali raised the yield to 70 %; it is thus apparent that the theoretical quantity of alkali is insufficient to ensure the completion of the reaction between benzyl chloroformate and the copper complex of ornithine. δ-Carbobenzyloxy-L-ornithine methyl ester hydrochloride was prepared by the first method of Synge (1948). The pure ester hydrochloride melted at 141° (uncorr.) $[\alpha]_D^{19°} + 15{\cdot}6°$ in methanol (*c*, 3·0); Synge (1948) reported melting point 132–134°, $[\alpha]_D^{19°} + 14–15°$ in methanol (*c*, 4·0). Carbobenzyloxyornithine was not coupled directly to the isomeric tripeptide esters, but was first combined with valine. L-Valine methyl ester hydrochloride prepared in this laboratory by the method of Fischer (1901) melted at 170°, whereas Synge (1948) reported melting point 146–149°.

The synthesis of α-(carbobenzyloxyvalyl)-δ-carbobenzyloxyornithine methyl ester by the azide method proved to be unexpectedly difficult. When carbobenzyloxy-L-valine azide was allowed to react at 0° with δ-carbobenzyloxy-DL-ornithine methyl ester either in ethyl acetate or in ether, the product was an intractable gel. Repeated fractionation of this gel eventually gave a low yield of the desired crystalline product. We suspected at first that our difficulty arose from the use of DL-ornithine instead of L-ornithine, but later, after we had prepared pure L-ornithine by the action of arginase on L-arginine, we were able to show that, in the reaction of carbobenzyloxy-L-valine azide with δ-carbobenzyloxy-L-ornithine methyl ester, the product was equally intractable and the yield equally bad. In the majority of peptide syntheses studied, the azide method has been the preferable method, but in this case the acid chloride method (Synge, 1948) using tosyl valine proved to be much superior.

α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithine hydrazide prepared from DL-ornithine was used for coupling with the isomeric tripeptides already synthesized (Harris & Work, 1950). It would have been desirable to use for this synthesis a dipeptide prepared from L-ornithine, but we preferred to reserve our limited supply of L-ornithine for the synthesis of the acylated pentapeptide amide which was to be compared with gramicidin S. Moreover,

we considered it unlikely that the use of DL-ornithine would invalidate our argument with regard to the significance of the optical configuration of phenylalanine. Both α-(*carbobenzyloxy*-L-*valyl*)-δ-*carbobenzyloxy*-DL-*ornithyl*-L-*leucyl*-L-*phenylalanyl*-L-*proline ethyl ester* and α-(*carbobenzyloxy*-L-*valyl*)-δ-*carbobenzyloxy*-DL-*ornithyl*-L-*leucyl*-D-*phenylalanyl*-L-*proline methyl ester* were difficult to purify and all attempts at crystallization resulted in the production of stiff gels. After repeated fractional precipitation of the amorphous compounds, correct analyses were obtained for the respective acylated pentapeptide esters and, as a check on their identity, an acid hydrolysate of each was subjected to chromatographic analysis on paper; the presence of the required amino-acids was demonstrated by comparison with an artificial mixture. Although in each case the final product gave almost theoretical analytical figures, we do not regard this as an adequate criterion of purity, and the physical constants quoted for the dicarbobenzyloxypentapeptide esters must be regarded as probable rather than absolute values.

The synthesis of an acylpentapeptide amide with a free δ-amino group on the ornithine residue required the preparation from valine and ornithine of an intermediate acyldipeptide ester with the α-NH_2 of valine and the δ-NH_2 of ornithine protected by groups of different stability. The *p*-toluenesulphonyl ('tosyl') group had a double advantage for this purpose; it was sufficiently stable to be unaffected by the conditions used for removal of a carbobenzyloxy group, and in addition it facilitated crystallization. Tosyl-L-valine was coupled with δ-carbobenzyloxy-L-ornithine methyl ester by the acid chloride method. The product, α-(*tosyl*-L-*valyl*)-δ-*carbobenzyloxy*-L-*ornithine methyl ester*, resembled α-(carbobenzyloxy-L-valyl)-δ-carbobenzyloxy-L-ornithine methyl ester in having zero rotation.

The conversion of α-(tosyl-L-valyl)-δ-carbobenzyloxy-L-ornithine methyl ester to the related hydrazide required exceptionally drastic conditions, but an excellent yield of hydrazide was eventually obtained by heating the ester under reflux with excess of 100% hydrazine hydrate in anhydrous methanol. The acylated dipeptide hydrazide was converted to the azide which coupled readily with L-leucyl-D-phenylalanyl-L-proline methyl ester to give α-(*tosyl*-L-*valyl*-δ-*carbobenzyloxy*-L-*ornithyl*-L-*leucyl*-D-*phenylalanyl*-L-*proline methyl ester*. The crude product resembled the corresponding carbobenzyloxypentapeptide ester in physical properties and tended to set to a stiff gel in all solvents. Crystallization was ultimately effected by extremely slow cooling of a saturated solution, the rate of cooling being controlled by the use of a large Dewar flask (see Experimental section). The final product was in the form of fine needles (Pl. 3*a*). Identity was checked by elementary analysis and by hydrolysis of the compound to its constituent amino-acids, which were then compared chromatographically with an artificial mixture (Pl. 3*b*).

As far as can be ascertained, this is the first recorded synthesis of a crystalline derivative of a pentapeptide built from five different optically pure amino-acids. In order to complete the series of intermediates, a tetrapeptide was synthesized by the coupling of αδ-dicarbobenzyloxy-L-ornithine with L-leucyl-D-phenylalanyl-L-proline methyl ester. The azide method was used for this synthesis.

The tosyl-carbobenzyloxy-pentapeptide ester was converted to the corresponding amide by reaction with ammonia. Selective removal of the carbobenzyloxy group was achieved by catalytic hydrogeneration of the amide. Selective removal by hydrogenation of the ester was also effected. The method of removal of the tosyl group to give the free pentapeptide will be reported in a later communication.

Since δ-carbobenzyloxy-L-ornithine was already available from the above synthesis we sought to couple it with phenylthiocarbonyl-L-valyl chloride. The phenylthiocarbonyl group can be removed under conditions which do not destroy the carbobenzyloxy group (Ehrensvärd, 1947), and in this way we expected to be able to synthesize a pentapeptide possessing a free α-NH_2 on the valine residue and an acyl group on the δ-NH_2 or ornithine. The addition of phenylthiocarbonyl-L-valyl chloride to two equivalents of δ-carbobenzyloxy-L-ornithine methyl ester resulted in rapid spontaneous removal of the protective phenylthiocarbonyl group. Apparently the alkalinity of the ester was sufficient to rupture the unstable thiocarbonyl linkage. *Methyl phenylthiocarbonyl*-L-*valyl*-p-*aminobenzoate* was successfully synthesized from phenylthiocarbonyl-L-valyl chloride and methyl *p*-aminobenzoate. In this case the aromatic amino group is much less basic and does not destroy the phenylthiocarbonyl group.

Antibacterial activity of products

The tetrapeptide ester, L-*ornithyl*-L-*leucyl*-D-*phenylalanyl*-L-*proline methyl ester* and the isomeric pentapeptide esters α-(L-valyl)-DL-ornithyl-L-leucyl-L-phenylalanyl-L-proline ethyl ester and α-(L-valyl)-DL-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester were tested for antibacterial activity, *in vitro*, against *Staphylococcus aureus*, *Streptococcus haemolyticus* and *Escherichia coli*. As can be seen from Table 1, the tetrapeptide ester and the two pentapeptide esters showed only limited activity, and the pentapeptide ester containing D-phenylalanine was not significantly more active than its isomer. The presence of the ester group in these peptides seems to have little influence on activity; thus,

(*a*)

(*b*)

(*a*) Photomicrograph by phase contrast of tosyl-L-valyl-δ-carbobenzyloxy-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester (× 1300).

(*b*) Paper-strip chromatogram of acid hydrolysate of tosyl-L-valyl-δ-carbobenzyloxy-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester (*A*) run simultaneously on Whatman no. 4 paper with an artificial mixture (*B*) of the component amino-acids; solvent system, 'collidine'-water). Ninhydrin was used for colouring the chromatogram. The yellow colour due to proline did not photograph well. Order of marked spots (from above downwards) ornithine, proline, valine, leucine, phenylalanine.

J. I. HARRIS AND T. S. WORK—THE SYNTHESIS OF PEPTIDES RELATED TO GRAMICIDIN S AND THE SIGNIFICANCE OF OPTICAL CONFIGURATION IN ANTIBIOTIC PEPTIDES. 2. PENTAPEPTIDES

Table 1. *Antibacterial activity of synthetic peptides related to gramicidin* S

(Bacteriostatic concentrations are given in the form $x \times 10^{-y}$M; the media were as specified in Part 1 (Harris & Work, 1950).)

Substance	*Streptococcus haemolyticus* Blood	*Streptococcus haemolyticus* Broth	*Staphylococcus aureus* Broth	*Escherichia coli* Broth
L-Ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester	—	2×10^{-3}	5×10^{-3}	4×10^{-3}
α-(L-Valyl)-DL-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester	$>1{\cdot}7 \times 10^{-3}$	$1{\cdot}7 \times 10^{-3}$	$>2{\cdot}0 \times 10^{-3}$	$>1{\cdot}7 \times 10^{-2}$
α-(L-Valyl)-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline	—	$3{\cdot}5 \times 10^{-3}$	—	—
α-(L-Valyl)-DL-ornithyl-L-leucyl-L-phenylalanyl-L-proline ethyl ester	—	$3{\cdot}5 \times 10^{-3}$	7×10^{-3}	$>7 \times 10^{-3}$
α-(Tosyl-L-valyl)-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester	$>1{\cdot}25 \times 10^{-4}$*	$>1{\cdot}25 \times 10^{-4}$*	$>1{\cdot}25 \times 10^{-4}$*	$>1{\cdot}25 \times 10^{-4}$*
α-(Tosyl-L-valyl)-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline amide	$>1 \times 10^{-4}$*	$>1 \times 10^{-4}$*	$>1 \times 10^{-4}$*	$>1 \times 10^{-4}$*
Gramicidin S	—	5×10^{-6}†	—	—

* Saturated solution. † Calculated on basis of cyclic pentapeptide.

a specimen of α-(L-valyl)-ornithyl-L-leucyl-D-phenylalanyl-L-proline prepared during our study on polymerization of pentapeptides (Harris & Work, unpublished) was found indistinguishable biologically from its ester.

α-(Tosyl-L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester hydrochloride and α-(tosyl-L-valyl)-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline amide hydrochloride were sparingly soluble in water and saturated solutions contained 10 and 8 mg./100 ml. respectively; no antibacterial activity was detected at these concentrations, whereas a sample of pure gramicidin S (kindly supplied by Dr L. C. Craig of the Rockefeller Institute, New York) was active at less than one-twentieth of these concentrations.

From the results given in Table 1 it is apparent that open-chain pentapeptides having the gramicidin S sequence of amino-acids possess only limited antibiotic action; in this respect they resemble the optically isomeric tripeptide fragments studied previously (Harris & Work, 1950). Activity does not appear to be directly related to the presence of amino-acids of 'unnatural' D-configuration since there is no appreciable difference in antibacterial potency between a pentapeptide incorporating D-phenylalanine in its structure and the corresponding peptide synthesized from L-phenylalanine.

The open chain pentapeptides differ from gramicidin S in that they possess reactive end groups (amino and carboxyl) in addition to the reactive amino group of the ornithine residue. As is evident from the formulae, synthetic α-(tosyl-L-valyl)-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline amide (I) bears a close resemblance to the natural antibiotic (II); the only reactive group is the δ-amino of the ornithine residue, but the presence of this polar group in an otherwise 'closed' pentapeptide chain having the correct amino-acid sequence and optical configuration is apparently not the key to activity as was envisaged by Znamenskaya, Agatov & Belozerskiĭ (1948).

On the basis of the chemical and physical evidence so far available, gramicidin S is best formulated as a *cyclic* pentapeptide or decapeptide (Consden, Gorden, Martin & Synge, 1947; Pedersen & Synge, 1948). The experimental results presented in the present paper furnish support for the postulate that the biological aetivity of gramicidin S is intimately related to its *cyclic* structure. Further discussion of this point is reserved for a future communication.

EXPERIMENTAL

Preparative studies

Melting points are given uncorrected; optical rotations were measured in a 4 dm. tube.

*δ-Carbobenzyloxy-*DL*-ornithine.* DL-ornithine was synthesized from acrylonitrile by the acetamidomalonate method of Albertson & Archer (1945), and converted to the δ-carbobenzyloxy derivative by the method described by Neuberger & Sanger (1943) for the preparation of ε-carbobenzyloxylysine. From ornithine monohydrochloride (13 g.), *δ-carbobenzyloxy-*DL*-ornithine* (9·6 g.), m.p. 256°, was obtained in the form of needles. (Found: C, 58·3; H, 6·6; N, 10·7. $C_{13}H_{18}O_4N_2$ requires C, 58·6; H, 6·75; N, 10·5 %.) *αδ-Dicarbobenzyloxy-*DL*-ornithine* (3·2 g.), m.p. 112°, was isolated from the reaction product; this indicated incomplete stability of the Cu complex of ornithine under the conditions used. (Found: C, 63·2; H, 6·4; N, 7·3. $C_{21}H_{24}O_6N_2$ requires C, 63·0; H, 6·0; N, 7·0 %.)

*δ-Carbobenzyloxy-*DL*-ornithine methyl ester.* δ-Carbobenzyloxy-DL-ornithine (12 g.) in anhydrous methanol

(120 ml.) was saturated with dry HCl gas at room temperature, and left for 24 hr. Concentration *in vacuo* below 30° gave the ester hydrochloride as a syrup. δ-Carbobenzyloxy-DL-ornithine methyl ester hydrochloride was not crystallized. It was converted to the free ester by the usual procedure.

L-Valine methyl ester hydrochloride. L-Valine (20 g.) was converted to the ester hydrochloride by the standard procedure of Fischer (1901). L-Valine methyl ester hydrochloride crystallized from ethanol-ether, and was recrystallized from methanol-ether in the form of lustrous rectangular plates, m.p. 170°. (Found: C, 43·1; H, 8·6; N, 8·0. Calc. for $C_6H_{13}O_2N.HCl$: C, 43·0; 8·4; N, 8·4 %.)

Carbobenzyloxy-L-valine hydrazide. Carbobenzyloxy-L-valine methyl ester (Synge, 1948) (14·0 g., m.p. 56°, prepared from the ester hydrochloride) was dissolved in methanol (100 ml.) and excess hydrazine hydrate (50 % (w/v), 14 ml.) was added. The mixture, left at room temperature for 24 hr., deposited *carbobenzyloxy-L-valine hydrazide* (13·8 g.) which was recrystallized from ethyl acetate-methanol as needles (12 g.), m.p. 179°. (Found: C, 59·1; H, 7·2; N, 16·1. $C_{13}H_{19}O_3N_3$ requires C, 58·9; H, 7·1; N, 15·9 %.)

α-(Carbobenzyloxy-L-valyl-δ-carbobenzyloxyornithine methyl ester. Carbobenzyloxy-L-valine hydrazide (6 g.) in a mixture of glacial acetic acid (50 ml.) and 2N-HCl (100 ml.) was cooled to 0°, and a solution of $NaNO_2$ (1·8 g.) in water (20 ml.) was added dropwise to the stirred solution during 15 min. The acid-insoluble azide was extracted into ice-cold ethyl acetate (150 ml.) and washed successively with ice-cold water and a cold saturated solution of $NaHCO_3$ until the washings were neutral to litmus. The azide solution was dried quickly over Na_2SO_4 and added during 30 min. to a cooled solution of δ-carbobenzyloxy-DL-ornithine methyl ester (7·8 g.) in ethyl acetate (50 ml.). A gelatinous precipitate was formed almost immediately, and after 24 hr. at 0° the whole solution had set to a stiff gel; this was collected by filtration and dried at 80° to give a white amorphous solid (9 g.), m.p. 150–160°. After several precipitations from ethyl acetate a crystalline product was obtained; three recrystallizations from ethanol gave α-(carbobenzyloxy-L-valyl)-δ-carbobenzoxy-ornithine methyl ester as clusters of small needles (1 g.), m.p. 150°, $[\alpha]_D^{17°} + 11·5°$ in $CHCl_3$ (*c*, 1·6). (Found: C, 62·6; H, 6·8; N, 8·3. Calc. for $C_{27}H_{35}O_7N_3$: C, 63·1; H, 6·9; N, 8·2 %.) In view of the racemic nature of the ornithine used a definite optical form cannot be assigned to this compound.

α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithine hydrazide. To a solution of the methyl ester (0·9 g.) in hot methanol (20 ml.) was added excess 90 % hydrazine hydrate (2 ml.); after 24 hr. at 37°, *α-(carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithine hydrazide* was precipitated as an amorphous gel which was collected and dried at 80° (0·75 g.). (Found: C, 60·6; H, 6·7; N, 13·9. $C_{26}H_{35}O_6N_5$ requires C, 60·8; H, 6·9; N, 13·6 %.)

α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithyl-L-leucyl-L-phenylalanyl-L-proline ethyl ester. The hydrazide (0·35 g.) was converted to the azide and extracted into ethyl acetate by the standard procedure already described; this azide was allowed to react at 0° with L-leucyl-L-phenylalanyl-L-proline ethyl ester (0·95 g.) dissolved in anhydrous ethyl acetate (50 ml.). After 24 hr. at 0° and 24 hr. at room temperature, excess tripeptide ester was extracted with N-HCl, and the ethyl acetate layer washed successively with water and saturated aqueous $NaHCO_3$. Removal of solvent under reduced pressure left a pale yellow viscous oil (0·53 g.) which was precipitated as gel, m.p. 124°, from a mixture of ethyl acetate and light petroleum. Several further precipitations from ethyl acetate-light petroleum gave a product (0·4 g.), m.p. 128°, which analysed correctly for *α-(carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithyl-L-leucyl-L-phenylalanyl-L-proline ethyl ester.* (Found: C, 65·0; H, 7·4; N, 9·8. $C_{48}H_{64}O_{10}N_6$ requires C, 65·1; H, 7·3; N, 9·5 %.) The presence of the expected amino-acids in the product was confirmed by paper chromatography of a hydrolysate.

α-(L-Valyl) ornithyl-L-leucyl-L-phenylalanyl-L-proline ethyl ester. The dicarbobenzyloxy derivative (335 mg.) was hydrogenated in methanol containing 2 equiv. HCl. *L-Valylornithyl-L-leucyl-L-phenylalanyl-L-proline ethyl ester hydrochloride* was obtained as an extremely hydroscopic semi-solid which was not crystallized.

α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester. The method used was similar to that described above for the synthesis of the isomeric pentapeptide derivative. α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithine hydrazide (0·35 g.) was converted to the azide and coupled with L-leucyl-D-phenylalanyl-L-proline methyl ester (0·9 g.) in ethyl acetate at 0°. *α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester* was obtained as a colourless, viscous oil which gave a white amorphous solid (400 mg.), m.p. 198–200°, from ethyl acetate-light petroleum. (Found: C, 64·5; H, 7·1; N, 10·0. $C_{47}H_{62}O_{10}N_6$ requires C, 64·8; H, 7·2; N, 9·7 %.) The presence of the expected five amino-acids was confirmed by paper chromatography after acid hydrolysis.

α-(L-Valyl) ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester. The dicarbobenzyloxy compound was hydrogenated in methanol containing 2 equiv. HCl, and the pentapeptide ester dihydrochloride isolate as a colourless, hygroscopic syrup (160 mg.) which could not be crystallized.

L-Ornithine. L-Arginine monohydrochloride was isolated from a protein hydrolysate by the flavianic acid method of Cox (1928), and converted into L-ornithine monohydrochloride by the arginase method of Hunter (1939).

Extraction and purification of arginase. A concentrate of arginase was obtained by a modification of the method described by Bach (1946). Fresh ox liver (2·5 kg.) was minced and treated with 5 vol. (10–12 l.) acetone at room temperature. The resulting suspension was filtered, the fibrous residue thoroughly macerated with another 2 vol. (5 l.) of acetone, and filtered again. The fibrous residue was air-dried at room temperature (750 g.). This product (300 g.) was then thoroughly macerated with 0·01 N-KOH (500 ml.); a further 1500 ml. KOH solution was added and the suspension stirred for 1 hr. The resulting gelatinous suspension was filtered at 0° and the precipitate washed with 0·002 N-KOH. The combined filtrates and washings (3700 ml.) were adjusted to pH 6·8 with HCl and heated in 500 ml. quantities at 54° for 5 min., with gentle shaking. The gelatinous suspension was adjusted to pH 6·1, cooled in ice, centrifuged, and the supernatant liquid was collected (3500 ml.); this liquid was cooled to 0° and the pH readjusted to 6·8. Cold acetone (1·2 vol.; 4200 ml.) was then added with stirring and the mixture left at 0° for 30 min. Adjustment to pH 6 with a few drops of HCl facilitated flocculation of the precipitate, which was centrifuged; the gelatinous protein precipitate was redissolved in the minimum volume of distilled water, and this solution (500 ml.) was treated at 0° with 1·2 vol. (600 ml.) of cold acetone. After keeping at 0° for 1 hr. the pH was adjusted to 6·2 and the resulting enzyme precipitate

collected by centrifugation. The enzyme precipitate dissolved in glass-distilled water (160 ml.) was stored at 0°.

Arginase activity was estimated by the Warburg manometric technique using the arginase-urease method developed by Hunter & Dauphinee (1930). In this way the activity of the arginase solution prepared as above was found to be 300 Hunter units/ml., giving a yield of arginase of approximately 50,000 Hunter units from 1 kg. liver.

From L-arginine monohydrochloride (30 g.), following the procedure of Hunter (1939), L-ornithine monohydrochloride (16·2 g.), $[\alpha]_D^{18°}$ +12·02° in water (*c*, 4·0), was obtained in 70 % yield.

δ-Carbobenzyloxy-L-ornithine methyl ester. Selective δ-acylation was effected in improved yield by slight modifications of the method used by Synge (1948). L-Ornithine monohydrochloride (5 g.) was converted to the complex copper salt and treated with 1 equiv. 2N-NaOH (15 ml.) at 0°. The resulting deep blue solution was treated with 2N-NaOH (25 ml.; 1·6 equiv.) and benzyl chloroformate (4·5 ml.) added alternately in ten equal portions during 30 min. at 0°. The mixture was stirred at room temperature for another 30 min. and the pale blue precipitate collected by filtration and washed with water and ethanol. After drying, the precipitate was finely powdered, suspended in water (500 ml.) containing HCl (40 ml. 2N), stirred mechanically and decomposed by a stream of H_2S. Precipitated CuS was removed by filtration, and washed thoroughly with hot water.

δ-Carbobenzyloxy-L-ornithine (6·1 g.) was precipitated from the combined filtrate and washings by adding NaOH (40 ml. 2N), and recrystallized from 50 % (v/v) aqueous ethanol, m.p. 254°; yield 5·6 g., 70 % of theoretical.

The methyl ester hydrochloride was obtained in almost quantitative yield by the method described by Synge (1948). Thus, δ-carbobenzyloxy-L-ornithine (5 g.) gave the corresponding methyl ester hydrochloride (5·35 g.) in the form of needles, m.p. 140–141° $[\alpha]_D^{19°}$ +15·6° in methanol (*c*, 3·0).

α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxy-L-ornithine methyl ester. Carbobenzyloxy-L-valine hydrazide (1·1 g.) was converted to the azide and allowed to react at 0° with an ethyl acetate solution of δ-carbobenzyloxy-L-ornithine methyl ester (50 % mol. excess; 1·65 g. prepared from 2 g. of the ester hydrochloride). A white gelatinous solid (0·45 g.) which precipitated from the reaction mixture was removed and the acylated dipeptide ester obtained from the filtrate by the procedure already used in the isolation of the corresponding derivative of DL-ornithine. In this way a white solid (1·2 g.) was obtained which crystallized from ethyl acetate as fluffy needles (0·8 g.), m.p. 110–116°. The product had excessive N content and the separation of pure acylated dipeptide ester from impurities proved to be very difficult. Partial purification was achieved by passing a solution of the crude substance (0·8 g.) dissolved in a 20 % (v/v) solution of $CHCl_3$ in benzene (80 ml.) through a column of 'Celite 545' (diatomaceous earth supplied by Johns Manville Co., London, S.W. 1). Crystallization of one of the eluate fractions, from ethyl acetate, gave needles (0·15 g.), m.p. 150°, $[\alpha]_D^{19°}$ ±0° in $CHCl_3$ (*c*, 3·0), which analysed correctly as α-(carbobenzyloxy-L-valyl)-δ-carbobenzyloxy-L-ornithine methyl ester (Synge, 1948). (Found: C, 63·3; H, 6·9; N, 8·4. Calc. for $C_{27}H_{35}O_7N_3$: C, 63·1; H, 6·9; N, 8·2 %.)

αδ-Dicarbobenzyloxy-L-ornithine hydrazide. αδ-Dicarbobenzyloxy-L-ornithine was prepared from L-ornithine monohydrochloride (1 g.) by the method described by Sy[illegible] (1948). The dicarbobenzyloxy derivative (1·9 g.) which crystallized from $CHCl_3$-light petroleum as fluffy needles, m.p. 114°, was converted through the methyl ester to the hydrazide by the standard procedure. *αδ-Dicarbobenzyloxy-L-ornithine hydrazide* (1·7 g.) was obtained as an amorphous solid, m.p. 126–128°. (Found: C, 60·4; H, 6·4; N, 13·6. $C_{21}H_{26}O_5N_4$ requires C, 60·9; H, 6·3; N, 13·5 %.)

αδ-Dicarbobenzyloxy-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester. αδ-Dicarbobenzyloxy-L-ornithine hydrazide (1 g.) was converted to the azide and coupled with L-leucyl-D-phenylalanyl-L-proline methyl ester (1·4 g.) in ethyl acetate solution at 0°. A small amount of a white gelatinous product (0·1 g.) insoluble in ethyl acetate, formed during the hydrazide-azide conversion, appeared to be αδ-dicarbobenzyloxy-L-ornithine amide (cf. Prelog & Wieland, 1946). The reaction *product* was isolated by the usual procedure as a viscous semi-solid which gave a pale-yellow amorphous solid (1 g.), m.p. 86–88°, on trituration with ether. (Found: C, 64·8; H, 6·7; N, 9·4. $C_{42}H_{53}O_9N_5$ requires C, 65·4; H, 6·9; N, 9·1 %.)

L-Ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester. The dicarbobenzyloxy derivative (0·6 g.) was successfully hydrogenated in methanol containing 2 equiv. HCl. Removal of catalyst and concentration of solvent *in vacuo* gave a pale yellow, hygroscopic, amorphous solid (0·4 g.) which did not give satisfactory crystalline material. Provisional identification of the product was achieved by paper chromatography.

Phenylthiocarbonyl-L-valine. Phenylthiocarbonyl chloride was prepared by the method of Rivier (1907). Thiophenol (21 g.) gave phenylthiocarbonyl chloride (24 g.), b.p. 108°/18 mm. Hg, $d_{15°}$ 1·285.

L-Valine methyl ester (2·8 g.) was dissolved in anhydrous ether (40 ml.) and phenylthiocarbonyl chloride (0·5 equiv.; 1·85 g.) in ether (30 ml.) added, with stirring, at room temperature during 5 min. according to the general procedure of Ehrensvärd (1947). After a further 15 min. L-valine methyl ester hydrochloride (2 g.) was collected by filtration; the filtrate was washed with N-HCl and water, dried and concentrated under reduced pressure. *Phenylthiocarbonyl-L-valine methyl ester* crystallized from light petroleum in the form of needles, m.p. 60–62°. Recrystallization from *cyclo*hexanol gave clusters of needles, m.p. 64°; yield 2·3 g. (80 % of theoretical). (Found: C, 58·2; H, 6·4; N, 5·1. $C_{13}H_{17}O_3NS$ requires C, 58·4; H, 6·4; N, 5·2 %.)

The methyl ester (2·1 g.) was heated on a boiling water bath for 20 min. in a 1:1 (v/v) mixture of conc. HCl and glacial acetic acid (20 ml.). Water (40 ml.) was added to the cooled solution and *phenylthiocarbonyl-L-valine* separated as an oil which solidified on standing at 0°. The solid was crushed, washed with water and dried (1·2 g.); concentration of the combined filtrates and washings gave a second crop (0·6 g.), and recrystallization of the combined products from ethyl acetate-ligroin yielded prisms (1·6 g.), m.p. 114°. (Found: C, 56·9; H, 6·13; N, 5·5. $C_{12}H_{15}O_3NS$ requires C, 56·9; H, 6·0; N, 5·5 %.)

Phenylthiocarbonyl-L-valyl-p-aminobenzoic acid methyl ester. Phenylthiocarbonyl-L-valine (0·5 g.) and 1 equiv. (0·44 g.) PCl_5 were suspended in anhydrous ether (10 ml.), and the mixture shaken at room temperature until all the solid material had dissolved. After 1 hr. 2 vol. light petroleum were added and, on cooling the solution below 0°, phenylthiocarbonyl-L-valyl chloride crystallized as needles, (0·35 g.), m.p. 66–68° (sealed tube).

The acid chloride (0·35 g.), dissolved in anhydrous $CHCl_3$ (5 ml.), was added dropwise, with shaking, to an excess of

methyl *p*-aminobenzoate in anhydrous ether (30 ml.), and the mixture left at room temperature overnight. *p*-Aminobenzoic acid methyl ester hydrochloride was collected and the filtrate was kept gently refluxing for 3 hr.; when cool, it was washed successively with N-HCl, saturated aqueous $NaHCO_3$ and water, dried over Na_2SO_4 and concentrated *in vacuo*. The pale yellow *residue* (0·25 g.) was crystallized in the form of white needles (0·15 g.), m.p. 170°. (Found: C, 61·8; H, 5·6; N, 7·5. $C_{20}H_{22}O_4N_2S$ requires C, 62·2; H, 5·7; N, 7·2 %.)

N-p-*Toluenesulphonyl*-L-*valine* (*tosyl*-L-*valine*). To a solution of L-valine (5 g.) in N-NaOH (55 ml.) was added solid *p*-toluenesulphonyl chloride (11 g.); the mixture was stirred vigorously at room temperature for 3 hr. Excess acid chloride was removed by filtration and the filtrate acidified to congo red with dilute HCl. Tosyl-L-valine (Karrer & Veer, 1932), which separated as a white crystalline precipitate, was collected, washed with water and dried. Crystallization from a mixture of ethyl acetate-light petroleum yielded rectangular plates (6·8 g.), m.p. 144°. (Found: C, 53·4; H, 6·1; N, 5·2. Calc. for $C_{12}H_{17}O_4NS$; C, 53·1; H, 6·3; N, 5·2 %.) As there was some danger of racemization in this preparation, a sample of the product was reduced by Na in liquid NH_3 and the valine isolated. The crude valine, as isolated, had a rotation close to that of L-valine.

Tosyl-L-*valyl*-*phenylalanine methyl ester*. Tosyl-L-valine (0·6 g.) and 1 equiv. PCl_5 (0·46 g.) were suspended in anhydrous ether (10 ml.), and the mixture was shaken at room temperature until all the solid material had dissolved. After 1 hr. the ethereal solution was concentrated and 2 vol. dry light petroleum added; tosyl-L-valyl chloride crystallized on cooling the solution below 0° in the form of thin needles (0·4 g.), m.p. 63°.

A solution of this acid chloride (0·4 g.) in ether (20 ml.) was added dropwise with shaking to an ethereal solution of DL-phenylalanine methyl ester (0·7 g., 2 equiv.), and the mixture left at room temperature overnight. DL-Phenylalanine methyl ester hydrochloride (0·4 g.) was removed by filtration, and the filtrate washed free from acid, dried over Na_2SO_4 and concentrated *in vacuo*. The residue (0·4 g.) crystallized from ethyl acetate-light petroleum as white, fluffy needles of *tosyl*-L-*valyl*-*phenylalanine methyl ester* (0·3 g.), m.p. 138–139°. (Found: C, 61·2; H, 6·4; N, 6·8. $C_{22}H_{28}O_5N_2S$ requires C, 61·1; H, 6·5; N, 6·5 %.)

α-(*Tosyl*-L-*valyl*)-δ-*carbobenzyloxy*-L-*ornithine methyl ester*. An ethereal solution of tosyl-L-valyl chloride (0·8 g.) was added to a stirred and cooled solution of δ-carbobenzyloxy-L-ornithine methyl ester (1·7 g., 2 equiv.) in anhydrous ether. A white precipitate formed immediately, and the mixture was left overnight at room temperature to complete the reaction. The thick, white, crystalline precipitate was collected, washed with ether and dried (2·4 g.). Excess δ-carbobenzyloxy-L-ornithine methyl ester was recovered as the hydrochloride by washing the precipitate with water, and the water-insoluble residue (1·48 g.) was recrystallized from acetone-methanol to give the acylated dipetide ester (1·2 g.) in the form of fluffy *needles*, m.p. 187°, $[\alpha]_D^{20}$ ±0° in $CHCl_3$ (*c*, 1·8). (Found: C, 58·8; H, 6·5; N, 7·9. $C_{26}H_{35}O_7N_3S$ requires C, 58·6; H, 6·6; N, 7·9 %.) In large-scale preparations by the same method the yield was 77 %, calculated on the acid chloride taken.

α-(*Tosyl*-L-*valyl*)-δ-*carbobenzyloxy*-L-*ornithine hydrazide*. The conversion of the acylated dipeptide methyl ester to the hydrazide was unusually difficult. After heating under reflux with excess of 90 % aqueous hydrazine hydrate for 2 hr. the ester was recovered almost completely unchanged. The hydrazide was, however, formed by heating the ester (2·5 g.) in anhydrous methanol (50 ml.) under reflux, with 3 equiv. A.R. hydrazine hydrate (100 %) for 6 hr. The hydrazide (2·4 g.) separated as a crystalline solid from the hot solution, and recrystallization from *iso*propanol gave pure *material*, m.p. 226–227°. (Found: C, 56·3; H, 6·6; N, 12·9. $C_{25}H_{35}O_6N_5S$ requires C, 56·3; H, 6·6; N, 13·1 %.)

α-*Tosyl*-L-*valyl*)-δ-*carbobenzyloxy*-L-*ornithyl*-L-*leucyl*-D-*phenylalanyl*-L-*proline methyl ester*. α-(Tosyl-L-valyl)-δ-carbobenzyloxy-L-ornithine hydrazide (1 g.) was converted to the azide, and a solution of the azide in dry ethyl acetate was added during 15 min. to a cooled solution of L-leucyl-D-phenylalanyl-L-proline methyl ester (1·1 g.; 50 % mol. excess) also in dry ethyl acetate (25 ml.). The solutions were well mixed and left at 0° for 24 hr. A white gelatinous precipitate (0·18 g.), m.p. 200–202°, which had separated at 0° was removed by filtration, and after 24 hr. at room temperature the filtrate deposited a fine amorphous precipitate (0·8 g.), m.p. 204–205°. Excess tripeptide ester was recovered as the hydrochloride by extracting the filtrate with N-HCl. The amorphous product, m.p. 204–205°, was crystallized from methanol by a special technique. The substance was heated under reflux with methanol, filtered hot to remove a small amount of sparingly soluble impurity, and the filtrate left to cool slowly by immersing the securely stoppered containing flask in water at 50° in a large Dewar flask. This procedure ensured a slow steady rate of cooling over a period of 3 days, and in this way α-(*tosyl*-L-*valyl*)-δ-*carbobenzyloxy*-L-*ornithyl*-L-*leucyl*-D-*phenylalanyl*-L-*proline methyl ester* crystallized as ball-shaped clusters of fine microneedles (3·6 g.), m.p. 200° (Pl. 3*a*). (Found: C, 62·0; H, 7·0; N, 9·3; S, 3·9. $C_{46}H_{62}O_{10}N_6S$ requires C, 62·0; H, 7·0; N, 9·4; S, 3·6 %.)

A second preparation from α-(tosyl-L-valyl)-δ-carbobenzyloxy-L-ornithine hydrazide (1·14 g.) gave the acylated pentapeptide ester (1·4 g.), crystallized by the procedure described above, as aggregates of microneedles, m.p. 201°; identity of the product was confirmed by paper chromatography of a hydrolysate (Pl. 3*b*).

α-(*Tosyl*-L-*valyl*)-L-*ornithyl*-L-*leucyl*-D-*phenylalanyl*-L-*proline methyl ester monohydrochloride*. α-(Tosyl-L-valyl)-δ-carbobenzyloxy-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester (0·5 g.) was dissolved in methanol (100 ml.) containing 1 equiv. HCl. Hydrogenation was effected in the presence of Pd black and evolution of CO_2 was complete in 90 min. After removal of catalyst the filtrate was concentrated *in vacuo*; the oily residue was dissolved in methanol and crystallized by the cautious addition of ether, yielding *microneedles* (0·42 g.), m.p. 236° (decomp.), $[\alpha]_D^{18°}$ −94·6° in methanol (*c*, 1·0). (Found: C, 57·4; H, 7·36; N, 10·7; Cl, 4·3. $C_{38}H_{56}O_8N_6S$.HCl requires C, 57·5; H, 7·2; N, 10·6; Cl, 4·5 %.)

α-(*Tosyl*-L-*valyl*)-L-*ornithyl*-L-*leucyl*-D-*phenylalanyl*-L-*proline amide monohydrochloride*. The diacylpentapeptide methyl ester (0·25 g.) was dissolved in methanol previously saturated with anhydrous NH_3 at 0° (10 ml.), and the solution kept at 37° in a sealed tube for 72 hr. The solvent and excess NH_3 were removed *in vacuo*; the solid residue was redissolved in methanol containing 1 equiv. HCl, and hydrogenated in the presence of Pd black by the usual method. After removal of catalyst, the filtrate was concentrated and adjusted to pH 4; the amide hydrochloride crystallized in the form of fine needles, m.p. 256–260° (decomp.), on the

cautious addition of ether. Recrystallization from methanol-ether gave *microneedles*, m.p. 268–270° (decomp.). (Found: N, 13·0%. $C_{37}H_{55}O_7N_7S.HCl$ requires N, 12·6%.)

SUMMARY

1. Two optically isomeric open-chain pentapeptide esters have been synthesized. These peptides have the same sequence of amino-acid residues as gramicidin S.

2. A crystalline acylpentapeptide amide has been synthesized. This compound is structurally analogous to the natural antibiotic in that its constituent amino-acids are arranged in the same sequence and are of the same optical configuration as those of gramicidin S and that its only reactive group is the δ-NH_2 of the ornithine residue.

3. These and other newly synthesized peptides were tested, *in vitro*, for antibacterial activity.

4. The bearing of the results on the question of the origin of antibiotic activity in gramicidin S is discussed.

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Proteolytic Enzymes of *Clostridium welchii*

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Culture filtrates from *Clostridium welchii* Type A contain an enzyme, collagenase (called κ-toxin by Oakley, Warrack & van Heyningen, 1946), which dissolves native collagen, softens muscle and also attacks degraded collagen as in hide powder and 'azocoll' (hide powder coupled with a red dye). The partial purification and some biochemical properties of this enzyme were described by Bidwell & van Heyningen (1948) and by Bidwell (1949*a*); observations on its effect *in vivo* and its action on gelatin were made by Oakley, Warrack & Warren (1948).

Walbum & Reymann (1934) found that filtrates from a strain of the lamb dysentery bacillus (*Cl. welchii* Type B) liquefied gelatin optimally at pH 7. Oakley *et al.* (1948) showed that *Cl. welchii* Type B produced a substance which attacked hide powder, azocoll and gelatin, but appeared to be without effect on undegraded collagen and muscle; it was antigenic and was designated the λ-antigen of *Cl. welchii*. It was inhibited by a substance in normal horse serum which is not identical with the trypsin inhibitor (Bidwell, 1949*b*). The present paper shows that the λ-antigen of Oakley *et al.* (1948) has the properties of a proteolytic enzyme; it will be referred to as 'λ-enzyme'. Its partial purification and properties will be described and compared with those of collagenase and the 'secondary' enzyme formed by the treatment of collagenase preparations with mild alkali or heat (Bidwell, 1949*a*).

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METHODS

Estimation of enzymic activity. The ability of λ-enzyme preparations to disintegrate azocoll was determined in '*R* units' in terms of a standard dried preparation by measuring the amount of dye liberated from azocoll under standard conditions. The standard preparation was crude culture filtrate of *Cl. welchii* Type B grown on meat broth medium containing 1% Parke Davis peptone, which was preserved by drying from the frozen state. The standard preparation contained 0·2 *R* unit of λ-enzyme/mg. by definition. The

magnitude of the *R* unit was so chosen as to indicate approximately the same level of activity against azocoll as that of 1*Q* unit of collagenase (Bidwell & van Heyningen, 1948).

A standard curve was constructed as follows: 100 mg. amounts of 60-mesh azocoll (Oakley *et al.* 1946) were shaken for 30 min. at 37° with 10 ml. enzyme diluted in phosphate buffer-saline (equal vol. of M/15-phosphate buffer pH 7·4 and 1 % (w/v) NaCl). Undissolved azocoll was filtered off through Whatman no. 31 or 41 papers. The filtrate was diluted to a convenient depth of colour (usually 3–6 times) and matched in a visual colorimeter using a green filter (Ilford 604) against a standard colour (1 %, w/v) solution of methyl red in 60 % (v/v) ethanol as stock solution; diluted for use 1/100 in 0·1 N-HCl). The colorimetric ratio, standard methyl red/standard enzyme preparation, was plotted against the enzyme units used to give an activity curve from which the activity of unknown solutions could be obtained in terms of the standard preparation. Fig. 1 shows the standard activity curve for λ-enzyme; the curve for collagenase under the same conditions is also shown. The two curves are very similar.

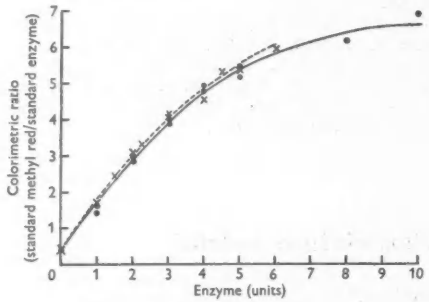

Fig. 1. Standard activity curves for the determination of the activity against azocoll of λ-enzyme (in *R* units) and collagenase (in *Q* units). Collagenase (*Q* units): - - × - - × - -. λ-Enzyme (*R* units): —●—●—. Varying amounts of enzyme in a total volume of 10 ml. phosphate buffer-saline (pH 7·4) were incubated with 100 mg. of azocoll (batch B478C) for 0·5 hr. at 37°. Colours of filtrates matched against 0·01 % methyl red in 0·1 N-HCl.

The ability of λ-enzyme preparations to hydrolyse casein and urea-denatured haemoglobin was determined by methods based on that of Anson (1938) for trypsin. Since this method is not applicable to very impure preparations on account of high blanks, a partially purified λ-enzyme preparation (B473E^{r}:57*R* units/mg.) was used as subsidiary standard. The substrates were 2·5 % (w/v) casein (Glaxo, L.W.S.) in veronal-acetate-HCl buffer (Michaelis, 1931) pH 7·1 and urea-denatured haemoglobin prepared according to Anson (1938). Enzyme (1 ml.) was added to 5 ml. substrate at 37°, mixed, incubated at 37° for exactly 10 min., 10 ml. 0·3 M-trichloroacetic acid added, shaken, and the mixture filtered after 30 min. (Whatman no. 3). In the controls trichloroacetic acid was added to the substrate before the enzyme. The 'tyrosine equivalent' of the filtrates was determined, the 'colour value' calculated and the enzyme unit fixed exactly as described by Anson (1938) as follows:

1·61 g. B473E^{r} = 1 enzyme unit, casein as substrate, [E.U.] casein,

2·56 g. B473E^{r} = 1 enzyme unit, haemoglobin as substrate, [E.U.] Hb.

Determinations of antibody combining power

λ-Antigen combining power values (Oakley *et al.* 1948) were determined by a method analogous to that used for κ-toxin combining power (Oakley *et al.* 1946). The standards fixed by Dr Oakley and Miss Warrack were sera refined by the pepsin process (Pope, 1938; 1939*a*, *b*) which destroys the non-specific λ-inhibitor of serum. The serum RX5417 was defined as containing 8000 anti-λ units/ml.; subsidiary standard RX7463 contained 1100 anti-λ units/ml.

Each of a series of tubes contained 10 units anti-λ antibody together with amounts of enzyme decreasing by 10 % in a total volume of approx. 2 ml. The mixture was allowed to stand 0·5 hr. at room temperature; 1 ml. of azocoll suspension (300 mg. to 100 ml.) was added to each tube. After incubation at 37° for 16–18 hr. the tests were read as described by Oakley *et al.* (1946).

λ-Combining power values using gelatin as substrate were determined by the gelatin-agar tube method of Oakley *et al.* 1948. Each test mixture contained 1 unit of an arbitrary standard serum (RR7480 ≡ 300 units/ml.).

β-Toxin combining power values were estimated by intracutaneous injection into guinea pigs (Glenny, Llewellyn-Jones & Mason, 1931); ε-toxin by the method of Batty & Glenny (1947) using as indicator the appearance of a specific necrotic reaction in rabbits. The antibody units were those in general use in these laboratories.

Other estimations. Total N was determined by the micro-Kjeldahl procedure; pH values were measured electrometrically using a glass electrode. Bacterial growth was estimated in terms of bacterial nitrogen as follows. Samples of the culture were centrifuged, the organisms washed twice with 0·05 % (v/v) acetic acid (Mueller, 1935) and total N determinations carried out.

Buffers. Where no reference is given the composition of the buffers used was that given by Clark (1928).

EXPERIMENTAL

Choice of medium. The first crude filtrate examined was from a medium which had been in general use in these laboratories for the growth of *Cl. welchii* Type B, a nutrient broth containing 1 % (w/v) Parke Davis peptone and meat and referred to as P.D. medium. This filtrate was highly active in disintegrating azocoll (10 *R* units of λ-enzyme/ml.), but later filtrates obtained with the same batch of medium were less satisfactory (5 *R* units/ml. or less). Since this batch of medium had been prepared with the last remnants of our stock of American peptone (irreplaceable under conditions then prevailing), it was essential to find an alternative medium. Many attempts were made to use one of a considerable variety of 'toxin media' available, and also to use media made according to the formula for P.D. medium but with other peptones, including a tryptic digest of meat, substituted for the Parke Davis brand, but all were unsuccessful (< 2 *R* units/ml., often < 1 *R* unit/ml.).

The effect of adding various substances to the medium. The basal medium for these experiments was made according to P.D. formula, but papain-digested horse muscle was used

in place of Parke Davis peptone. The addition of glucose resulted in an increase in growth but there was marked inhibition of λ-enzyme production. The addition of calcium or magnesium to the medium has been stated to promote production of bacterial protease although without effect on growth (Merill & Clark, 1928; Haines, 1931, 1932); there was no improvement in growth of *Cl. welchii* Type B on further addition of Ca or Mg to a basal medium containing initially 33 mg. Ca^{++}/l. and 19 mg. Mg^{++}/l. The addition of Mg had no effect on λ-enzyme production, but Ca (1–4 ml. M-$CaCl_2$/600 ml. medium) produced a small but definite rise (increase in *R* value up to 50 % over control). Since a precipitate (probably Ca phosphate) appeared in the medium on the addition of $CaCl_2$, the increase in λ-enzyme might have been due to the removal of inhibitory substances (e.g. trace metals) and not to an increase in Ca ion concentration. Part of a batch of medium was therefore 'deferrated' (i.e. a Ca phosphate precipitate was formed in the medium and removed by filtration), but no improvement resulted. The addition of asparagine (Diehl, 1919) was without effect.

Final medium. A pancreatic digest of casein (Adams & Hendee, 1945) was tried and found to be so successful for the production of λ-enzyme that no further work on meat digests was done. In the medium as finally used values of 10–20 *R* units of λ-enzyme/ml. were regularly obtained; occasionally even higher values have been found.

The medium was developed by Adams & Hendee (1945) for the production of high value *Cl. welchii* α-toxin. The casein is digested using a relatively large proportion of minced whole pancreas. Adams, Hendee & Pappenheimer (1947) have shown that the pancreas provides a toxigenic factor (for α-toxin production) apart from its function in digesting the casein. Adams & Hendee (1945) recommended insoluble dextrin as an energy source (see also Logan, Tytell, Danielson & Griner, 1945), but as this was not available, the less soluble fraction of a yellow dextrin was used in the preliminary experiments. Adams *et al.* (1947) stated that potato starch may be used instead of insoluble dextrin for the production of α-toxin; it was found to be rather better than the dextrin for λ-enzyme production, and was used in all subsequent work at a final concentration of 0·25 % (w/v). This amount of starch gave about 50 % more λ-enzyme than was obtained in the absence of starch.

It may be remarked that all our attempts to produce high value α-toxin by growing *Cl. welchii* Type A on this medium, for which it was originally designed, have been unsuccessful. Only in one instance was even a very low level of α-toxin attained, although we have used Adams & Hendee's (1945) own strain which they kindly sent to us as well as the classical S107. This may indicate that although we adhered closely to the directions given by Adams & Hendee (1945) the medium as prepared by us differs in some essential point from that of the originators, possibly due to a difference in the raw material. We used a crude casein obtained from Lactic Casein Importers Ltd. Remarks made here concerning this medium may not be applicable to the medium as prepared in Adams & Hendee's laboratory.

Inoculum. A 5 hr. culture (50 ml.) grown on ordinary meat broth medium containing meat was used for every litre of casein medium.

Time of growth. λ-Enzyme activity was already very marked after 4 hr. at 37°, reached a maximum at 5 hr. and stayed fairly constant up to 24 hr. Standard conditions: 5 hr. at 37°.

Strain. A few different strains of *Cl. welchii* Type B were tried, but most of the experiments quoted here were done with a strain (CN 1990, Wellcome Laboratories) isolated by Mrs J. M. Barnes in the spring of 1946 from the intestine of a lamb dead from a typical lamb dysentery infection.

Appearance of organisms. Stained culture smears of *Cl. welchii* Type B grown on Adams & Hendee's (1945) medium often presented a remarkable atypical appearance. The organisms were often unusually large and unevenly stained. Frequently, although not invariably, spores were seen, sometimes in very large numbers, even in the 5 hr. cultures. The occurrence of spores seemed to be unrelated to the fact that this medium promotes λ-enzyme formation. When other types of *Cl. welchii* were grown on this medium, the appearance of the organisms was normal.

RESULTS

Purification of λ-enzyme

The first attempts at purification were made using culture filtrates obtained on meat media. The precipitates obtained by adding ammonium sulphate (600 g./l.) did not dissolve readily in water, as our experience with *Cl. welchii* Type A had led us to expect. Instead the formation of a peculiar jelly which could be dissolved on warming at 37° was noted on several occasions. After dialysing against tap water, the material was readily soluble, but dialysis resulted in a marked increase in activity against azocoll (50–80 %) as if an inhibitor had been removed. Nutrient broth did not inhibit the action of λ-enzyme on azocoll so it appears that an inhibitor may be formed during the growth of the organism. These effects were not observed with culture filtrates obtained on the casein medium and the point was not pursued. The λ-enzyme could not be adsorbed on calcium phosphate (used for the purification of collagenase) nor could it be purified by the nucleic acid method which van Heyningen & Bidwell (1948) applied to *Cl. welchii* α-toxin.

Ammonium sulphate fractionation. The best preparation obtained by a series of three fractionations with ammonium sulphate when meat media were used contained 20–24 *R* units of λ-enzyme/mg. of non-dialysable nitrogen (NDN) representing a purification of only four- or five-fold. When the casein medium was used, the crude starting material already contained about 20 *R* units of λ-enzyme/mg. NDN and moreover, by the same procedure of ammonium sulphate fractionation, a further purification of 15- to 30-fold could be attained.

The method finally adopted was as follows:

Culture filtrates obtained after 5 hr. growth on casein medium were clarified by filtration through paper pulp with the aid of Hyflo Supercel (Johns-Manville Ltd.), sterilized by passing through bacteriological filter candles, and $(NH_4)_2SO_4$ (600 g./l.) stirred in. After standing overnight at 2–4°, as much as possible of the rather small precipitate was skimmed from the surface and the rest collected by filtration

using a small amount of Hyflo Supercel. The precipitate may be dialysed directly against running tap water. After overnight dialysis, the material was cooled to about 2° in an ice-water bath, 2 vol. ice-cold 51 % (w/v) $(NH_4)_2SO_4$ solution added very slowly with stirring, left overnight at 2–4° and the precipitate collected by filtration with the aid of Hyflo Supercel. The dialysis and precipitation with 2 vol. saturated $(NH_4)_2SO_4$ was repeated once. After a final dialysis, the material was usually preserved by drying from the frozen state.

An illustrative protocoll is given in Table 1 which represents an average purification, but not the best we attained. Table 2 shows that λ-antigen is retained through the stages of purification.

The purest preparation so far obtained contained 1630 *R* units/mg. non-dialysable nitrogen; overall yield was 42 %. The corresponding dried preparation contained 116 *R* units/mg.

For comparison, the standard dried preparation (0·2 *R* unit/mg.) contained 5 *R* units/mg. non-dialysable nitrogen; preparations made on ordinary meat broth medium were even more impure (approx. 1 *R* unit/mg. non-dialysable nitrogen). Incidentally to this work, the ε-toxin of *Cl. welchii* has been considerably purified, occurring in the same purified fraction as the λ-enzyme, although, had this been the object of the work, a culture filtrate from *Cl. welchii* Type D would probably have been chosen as starting material. The ε-toxin in purified preparations appeared to be fully activated, possibly by the λ-enzyme, since the test dose was not decreased by trypsin.

Properties of λ-enzyme

pH *stability*. A partially purified λ-enzyme preparation (750 *R* units/ml.; 9000 λ combining-power units/ml.; 420 *R* units/mg. NDN) was diluted 1/10 in 1 % (w/v) NaCl; samples were adjusted to varying pH values as shown in Table 3 using 0·1 N-NaOH or 0·1 N-HCl. Each, as prepared, was heated for 30 min. at 37°, then cooled and re-adjusted to pH 7. A precipitate was obtained in the sample at pH 4. The control preparation was kept at pH 7 and room temperature. The results are shown in Table 3. The [E.U.] casein value for the preparation heated at pH 7 is slightly higher than would have been expected from comparison with the other activities of the same preparation, but on the whole the activities are remarkably parallel. There is some suggestion that at pH values above 7, the active enzyme is less stable than total antigen, but at acid pH values enzymic activity and combining power are destroyed in parallel.

Heat stability of λ-enzyme. A sample of the preparation that was used in the previous experiment was diluted 1/10 in borate buffer pH 7·4 and divided into seven equal parts which were heated for exactly 10 min. to varying temperatures.

Table 4 shows that the purified enzyme is very heat labile, since about 75 % of the activity was destroyed by heating to 56° for 10 min. The loss in antibody combining power was parallel to the loss in enzymic activity. After 10 min. at 50°, about 20 %

Table 1. *Purification of λ-enzyme*

Stage no.	Description	λ-enzyme: *R* units/ml.	Stage yield (%)	*R* units/mg. NDN*	Overall purification†	mg. NDN*/ml.
1	Crude culture filtrate (A)	16·6	—	46	—	0·362
	23 l. A precipitated with $(NH_4)_2SO_4$ (600 g./l.); ppt. dissolved in water (B, 410 ml.)	539	67	207	4·5	2·60
	395 ml. B dialysed overnight (C; 730 ml.)	243	84	—	—	—
2	725 ml. *C* + 2 vol. 51 % (w/v) $(NH_4)_2SO_4$; ppt. dissolved in water (D; 228 ml.)	740	95	—	—	—
	225 ml. D dialysed overnight (E; 295 ml.)	508	89	674	14·7	0·753
3	50 ml. E + 2 vol. 51 % (w/v) $(NH_4)_2SO_4$; ppt. dissolved in water (F; 27·0 ml.)	865	90	—	—	—
	26·5 ml. F dialysed overnight (G; 30 ml.)	685	90	835	18·6	0·804

* NDN = non-dialysable nitrogen.

† Purification expressed in terms of non-dialysable nitrogen by the ratio $\frac{\text{Activity/mg. NDN of product}}{\text{Activity/mg. NDN of starting material}}$.

Table 2. *The comparison of the enzyme activity against azocoll (in R units/ml.) and the λ-antigen content (antibody combining power units/ml.) at successive stages of the purification of three different batches*

Stages of purification (see Table 1)	Ratio λ units*/ml. : *R* units/ml. Batch 1	Batch 2	Batch 3
Crude filtrate	6·5	8·4	5·4
1	7·6	4·3	—
2	6·1	3·4	4·6
3	8·4	7·0	7·9

* Antibody combining-power units.

Table 3. pH *stability of λ-enzyme*

(Each sample of enzyme was adjusted to a particular pH, heated 30 min. at 37°, cooled and readjusted to pH 7 before test.)

Substrate ...	Enzyme activities after treatment: Azocoll		Casein	Haemoglobin	Gelatin
pH	*R* units/ml.	λ units/ml.*	[E.U.] casein × 10^6/ml.	[E.U.] Hb × 10^6/ml.	Test doses/ml.*
4	27	294	350	200	33
5	61	720	—	—	—
6	70	770	—	—	—
7	66	740	1200	480	71
8	69	890	—	—	—
9	61	890	—	—	—
10	44	710	800	350	77
Control	79	870	1200	600	—

* Antibody combining-power units.

of the activity against casein and about 30 % of the activity against haemoglobin had been lost. After 10 min. at 56° the losses were 80 and about 70 % respectively, and this treatment also resulted in a loss of about 75 % of the combining power as measured by the gelatin agar test. After 10 min. at 60°, the enzyme activity in *R* units and the combining power (both azocoll and gelatin-agar) were all 5 % of the value of the control (20° sample).

Table 4. *Effect on the enzyme activity and combining power of λ-enzyme of heating for* 10 *min. at various temperatures*

Temp. (°)	*R* units/ml.	λ units/ml.*
Control (20)	70	820
45	64	710
50	50	680
56	17	160
60	3	30
70	<1	<2

* Antibody combining-power units.

Optimum pH. The experiments on the action of λ-enzyme preparation on hide powder were carried out as described for collagenase (Bidwell & van Heyningen, 1948). There was very little variation in activity over the range pH 5–8, with a very sharp drop below pH 5. The curves for λ-enzyme were similar to those obtained for the action of collagenase and the 'secondary' azocoll enzyme (Bidwell, 1949*a*) on hide powder. Collagen prepared by the method of Delaunay, Guillaumie & Delaunay (1949) was dissolved most rapidly by collagenase preparations at pH 6·5 (veronal-acetate-HCl buffer); the other enzymes were without effect. There was little variation in the activity of λ-enzyme preparations on casein over the range of pH 6–7·2 or on haemoglobin over the range 7–8·4.

Optimum substrate 'concentration' for the action of λ-enzyme preparations on hide powder. The difficulty of working with concentrated suspensions of hide powder which had been encountered in the experiments with collagenase was greatly accentuated with λ-enzyme. Although hide powder was readily dissolved, further breakdown appeared to be slow, so that, when the higher concentrations of hide powder were used, the mixture was extremely

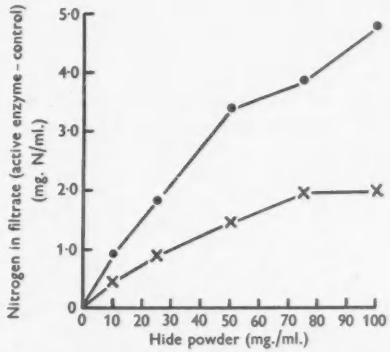

Fig. 2. Variation of enzyme activity with 'concentration' of hide powder in suspension. Collagenase, final concentration 0·28 *Q* unit/ml., in borate buffer, pH 7·4, — × — × —, λ-enzyme, final concentration 0·4 *R* units/ml., in borate buffer, pH 7, —●—●—. Varying amounts of hide powder in total volume 10 ml. buffer-enzyme mixture incubated for 1 hr. at 37°. Controls: heat-inactivated enzyme.

difficult to filter, being very 'gelatinous'. It was feared that results might be invalidated by the enzyme reaction continuing during the filtration and therefore the experiment was repeated with the modification that after incubation the mixture was rapidly heated to 70° for 10 min. to destroy the enzyme before filtering. This procedure resulted in slightly higher values in the controls, as might be expected, but the result did not differ qualitatively from that previously obtained for λ-enzyme. Fig. 2

shows that the results with λ-enzyme were very different from those obtained with collagenase, since even the highest concentration of hide powder attainable did not appear to be near the optimum for λ-enzyme.

Activity curve. Owing to the difficulty in filtering the digested hide powder after the action of the λ-enzyme and the fact that no optimum 'concentration' of substrate was reached, no attempt was made to determine an activity curve for the action of λ-enzyme on hide powder.

The action of λ-enzyme preparations on gelatin. *Cl. welchii* Type B filtrates contain a substance which attacks gelatin so that it no longer gives a precipitate on the addition of an acid solution of mercuric chloride. This substance is probably identical with λ-enzyme (Oakley *et al.* 1948). λ-Enzyme preparations attack gelatin with the formation of split products which are not precipitated on the addition of acidified phosphotungstic acid under the conditions described for collagenase (Bidwell & van Heyningen, 1948). The actions of partially purified preparations of collagenase, the 'secondary' enzyme and λ-enzyme on gelatin, as measured by the non-precipitable nitrogen method, are all considerably increased in the presence of horse serum.

It appeared possible that our enzyme preparations might contain a kinase capable of activating serum protease (compare streptokinase, Christensen, 1945, 1946; Christensen & MacLeod, 1945), but experiments designed to test this were all negative. The effects observed by this method may largely be due to yet another enzyme, possibly a polypeptidase which is activated by normal horse serum and is a relatively poor antigen.

Substrate specificity

Casein and haemoglobin. Substrates used were 2·5 % casein in veronal-acetate-HCl buffer, pH 7, and urea-denatured haemoglobin (Anson, 1938). 'Tyrosine equivalents' were determined on the filtrates by the method of Anson (1938). Table 5 shows that λ-enzyme preparations attacked both substrates; the results obtained with collagenase and the 'secondary' enzyme are included for comparison.

The question whether the splitting of casein and haemoglobin by λ-enzyme preparations can be regarded as due to λ-enzyme has not been satisfactorily settled. From a comparison of the activities of several samples at different stages of purification when tested against the different substrates it appeared that more than one enzyme must be involved. Normal horse sera and specific antisera inhibited the action of the preparations on casein and haemoglobin; we therefore tried to tackle the problem by serological methods. The results of attempts at the quantitative determination of inhibitory activity of sera using casein as substrate were however very inconsistent and were even less promising with haemoglobin.

Silk proteins. Raw silk or silk fibroin (400 mg.) cut into small pieces were used in a total volume of 20 ml. Where cysteine was used the final concentration was 0·004 M (pH adjusted to 7). Collagenase (10 *Q* units/ml.) and 'secondary' enzyme (5 '*Q*' units/ml.) preparations had no effect on raw silk in the absence of cysteine; with added cysteine there was an increase in filtrate nitrogen in 20 hr. at 37°. λ-Enzyme (10 *R* units/ml.) had a definite action on raw silk which was inhibited by cysteine (Table 6). None of the enzymes digested silk fibroin, either with or without cysteine, so the action on raw silk was probably due to digestion of seracin.

Clupein. Collagenase and λ-enzyme preparations showed an extremely small activity which was enhanced by cysteine; the more active preparation (λ) split only about 3 % of the available bonds after 20 hr. at 37°. The final concentration of enzyme in the solution was approx. 8 *Q* or *R* units/ml. The 'secondary' enzyme was completely inactive, even in the presence of cysteine.

For comparison either collagenase of λ-enzyme in a concentration of about 0·3 *Q* or *R* units/ml. will digest nearly 25 % of the hide powder present in 2·5 % suspension in 1 hr. at 37°.

Peptidase activity. Partially purified λ-enzyme preparations liberated titratable groups from gelatin, but their activity was even weaker than that of collagenase (Bidwell & van Heyningen, 1948). The relative weakness of λ-enzyme in breaking down the

Table 5. *Action of collagenase, 'secondary' enzyme and λ-enzyme on casein and haemoglobin*

(5·0 ml. substrate and 1 ml. enzyme solution were incubated at 37° for the times shown. 10 ml. 0·3 M-trichloroacetic acid were added and the mixtures filtered after 0·5 hr. Controls: precipitant added before enzyme.)

Enzyme	Activity against azocoll (*Q* or *R* units)	Substrate	Digestion time (min.)	Tyrosine in 5 ml. digestion products (m-equiv. × 10^4)
Collagenase	300	Casein	30	2·7
	450	Haemoglobin	30	2·0
'Secondary' enzyme	300	Casein	30	−0·4
	350	Haemoglobin	30	+0·8
λ-Enzyme	30	Casein	10	9·1
	45	Haemoglobin	10	10·7

Table 6. *The action of collagenase 'secondary' enzyme and λ-enzyme on silk proteins*

Enzyme	Cysteine	Substrate	N liberated (mg./5 ml. filtrate): Active enzyme	Heat-inactivated enzyme	Difference
Collagenase	–	Raw silk	0·145	0·124	0·021
	+	Raw silk	0·896	0·684	0·212
'Secondary'	–	Raw silk	0·075	0·070	0·005
	+	Raw silk	0·625	0·345	0·280
λ-Enzyme	–	Raw silk	0·599	0·128	0·471
	+	Raw silk	0·619	0·505	0·114
Collagenase	–	Fibroin	0·127	0·103	0·024
	+	Fibroin	0·469	0·447	0·022
'Secondary'	–	Fibroin	0·065	0·055	0·010
	+	Fibroin	0·325	0·315	0·010
λ-Enzyme	–	Fibroin	0·110	0·098	0·012
	+	Fibroin	0·470	0·474	−0·004

Table 7. *Effect of cysteine on the digestion of hide powder by* (a) *λ-enzyme*, (b) *collagenase*

(100 mg. hide powder, 9 ml. borate buffer and 1 ml. enzyme maintained at 37° for 0·5 hr. Filtered. Total N determined in filtrate.)

Enzyme	Cysteine	N liberated (mg./ml. filtrate): Active enzyme	Heat-inactivated enzyme	Difference	Inhibition (%)
λ-Enzyme	–	0·637	0·166	0·471	–
(0·21 *R* units/ml.)	+	0·235	0·212	0·023	>90
Collagenase	–	0·497	0·189	0·308	–
(0·17 *Q* units/ml.)	+	0·397	0·225	0·172	44

gelatin has already been noted in connexion with the experiments on hide powder (see also Walbum & Reymann, 1934).

Enzyme inhibitors

Metallic ions. The activity of λ-enzyme preparations in azocoll tests was almost completely destroyed by heating 0·5 hr. at 37° with 0·001 M solutions of copper or mercury salts; under the same conditions, ferric iron had no effect, while ferrous iron decreased the activity to about 50 %. The digestion of hide powder by λ-enzyme previously dialysed against distilled water was unaffected by the addition of calcium or magnesium ions in final concentrations of 0·1 or 0·001 M.

Cysteine. Equal volumes of enzyme preparation and 0·1 M-cysteine (adjusted to pH 7) were allowed to stand together for 10 min.; control mixtures were made containing saline instead of cysteine.

Table 7 shows that both λ-enzyme and collagenase are markedly inhibited by cysteine. It seems probable that the slight action of collagenase preparations on casein, haemoglobin, seracin and clupein which is increased in the presence of cysteine is not due to collagenase itself, but perhaps to traces of Maschmann's 'anaerobiase' (Maschamnn, 1938*a*, *b*)

Weil & Kocholalaty (1937) and Weil, Kocholaty & Smith (1939) stated that the gelatinase of *Cl. welchii* was markedly activated by cysteine + iron. Both collagenase and λ-enzyme showed decreased activity in dissolving hide powder in the presence of the concentrations of cysteine and iron used by Weil *et al.* (1939).

Iodoacetic acid (final concentration 1 mg./ml.) had a slight inhibitory effect on both enzymes. λ-Enzyme was slightly inhibited by cyanide, but collagenase was not affected at the concentration used (enzyme preparations were allowed to stand 0·5 hr. at room temperature with 0·01 M-cyanide); concentration of cyanide during activity test was approx. 0·0002 M).

Citrate. The inhibitory effect of citrate was tested using purified collagenase, before and after alkali treatment, and purified λ-enzyme, all three being diluted to approximately the same activity against azocoll (5*Q* or *R* units/ml.). Table 8 shows that neither collagenase nor the secondary enzyme was appreciably affected by a final concentration of 0·1 M-citrate, whereas λ-enzyme was 75 % inhibited.

'Trypsin inhibitors.' Soybean trypsin inhibitor was without effect on any of the three enzymes when tested against azocoll: equal volumes of enzyme (diluted to approx. 5*Q* or *R* units/ml.) and inhibitor solution (5 or 0·5 mg./ml.) were mixed, left 0·5 hr. and the activity against azocoll then determined and shown to be the same as when no soybean inhibitor was present. Pancreatic trypsin inhibitor was tested

Table 8. *The effect of citrate on the activity of collagenase, the 'secondary' enzyme and λ-enzyme in disintegrating hide powder*

(250 mg. hide powder and 10 ml. enzyme mixture maintained at 37° for 1 hr. (1) With citrate: 7 ml. borate buffer pH 7 + 2 ml. 0·5 M-citrate pH 7 + 1 ml. enzyme. (2) Without citrate: 9 ml. borate buffer pH 7 + 1 ml. enzyme. (3) Enzyme controls: corresponding heat-inactivated enzymes.)

Enzyme	mg. N/ml. filtrate: With citrate	mg. N/ml. filtrate: Without citrate	Difference (active enzyme control): With citrate	Difference (active enzyme control): Without citrate	Inhibition by citrate (%)
Collagenase	1·98	2·04	1·62	1·58	0
Collagenase control	0·36	0·46			
'Secondary' enzyme	2·07	2·10	1·72	1·63	0
'Secondary' control	0·35	0·47			
λ-Enzyme	0·74	2·12	0·40	1·64	75
λ-Enzyme control	0·34	0·48			

Table 9. *Comparison of the properties of collagenase, 'secondary' enzyme and λ-enzyme*

Observation	Collagenase	'Secondary' enzyme	λ-Enzyme
Occurrence in culture filtrates of types of *Cl. welchii*	Types A, C, E, some D	?	Types B, E, some D
Action on substrates:			
Native collagen	+	−	−
Hide powder, gelatin	+	+	+
Casein, haemoglobin	(−)	−	+
Seracin	(−)	(−)	+
Clupein	−	−	−
Activity *in vivo*	Various activities ascribable to attack on collagen (Oakley *et al.* 1948)	Probably none	None known
Optimum pH	pH 6·5 (collagen) pH 6·0–7·5 (hide powder)	pH 5·0–7·5	pH 6·0–7·5
Effect of acid	Unstable at pH <approx. 5	Unstable at pH <approx. 5	Unstable at pH <approx. 5
Effect of alkali	Unstable at pH >approx 8·5	Appears in collagenase preparation at pH between 9 and 10·5. Destroyed at pH >10·5	Unstable at pH > 9
Effect of heat	Destroyed in 10 min. at 56°	Appears in collagenase preparations at 50°, destroyed in 10 min. at 60° in borate buffer, probably more stable in distilled water	95 % destroyed in 10 min. at 60°
Inhibition by:			
Cysteine	+	?	+ +
Cyanide	−	?	+
Citrate	−	−	+
Normal horse serum	−	−	+

in a similar way, but owing to insufficient material being available only the lower concentration (0·5 mg./ml.) was used, and the effect on the secondary enzyme was not tested. λ-Enzyme was definitely not affected. The activity of the collagenase preparation was decreased about 10 %, but it was unfortunately impossible to repeat the experiment as no more inhibitor was available.

A comparison between the properties of λ-enzyme, collagenase and the 'secondary' enzyme (which appears in preparations of collagenase which have been treated with alkali or heat) is summarized in Table 9.

DISCUSSION

Maschmann (1937, 1938*a*, *b*) obtained a protease (anaerobiase) from culture filtrates of *Cl. welchii* which could split casein and gelatin (optimally only in the presence of cysteine). Clupein and peptone were also split if a further, unknown activator in addition to cysteine was present. Anaerobiase was inhibited by normal serum, but cannot be identified with λ-enzyme, since the latter is inhibited by cysteine even more markedly than is collagenase. It seems unlikely that any of our preparations has contained more than, at most, a trace of anaerobiase.

The enzyme we have called collagenase seems to be the same as the 'specific gelatinase' (collagenase) of Maschmann.

Maschmann (1938*c*) described a 'new' proteinase of *Clostridium sporogenes* (*B. sporogenes*) which appears to be similar to λ-enzyme in some of its biochemical properties, e.g. inhibition by citrate and normal horse serum; partial inhibition by cysteine and cyanide; optimum pH; lability to acid pH and its comparatively weak gelatinase action when assessed by a method depending on the degree of chemical degradation of the substrate.

An enzymic digest of casein probably simulates the natural medium of a lamb dysentery infection rather more closely than the conventional meat medium, so that a comparison of toxin production on the two types of media is of particular interest.

When grown on the ordinary laboratory meat media, *Cl. welchii* Type B usually produces high β-toxin and low ε-toxin values. (The average of thirteen different routine batches was 0·57 ml. = 1 ε unit of antitoxin; 0·58 ml. = 10 β units). With Adams & Hendee (1945) medium β values were very low (> 1·0 ml. = 2 β units) and ε values relatively high (usually 0·2–0·4 ml. = 5 ε units). *Cl. welchii* Type D is routinely grown on meat broth medium containing meat particles; the mean of seventeen such batches of Type D toxin gave 0·39 ml. = 5 ε units, i.e. Type B grown on casein medium can produce as much ε-toxin as Type D. On a few occasions Type D was grown on casein medium; the ε values were no higher than observed with Type B. It seems likely that the low β values were due to destruction of β-toxin by a proteolytic enzyme (such as λ-enzyme) since when a strain of Type C (which does not produce λ-enzyme) was grown, a titre of about 0·1 ml. = 10 β units was obtained, and the growth of Type B in the presence of normal horse serum (which inhibits λ-enzyme) resulted in the production of small amounts of β-toxin. Thus, apart from the high λ-enzyme content of filtrates obtained on the casein medium, the content of β- and ε-toxins is similar to that obtained on meat media with Type D strains rather than Type B. Unpublished experiments suggest that the unusual distribution obtained on Adams & Hendee's (1945) medium arises from some factor in whole pancreas which is not extracted by the usual processes for preparing 'pancreatic extract'.

SUMMARY

1. The partial purification and properties of the λ-enzyme of *Clostridium welchii* Type B are described. In a special medium (pancreatic digest of casein) *Cl. welchii* Type B produced high yields of λ-enzyme and ε-toxin, but little or no β-toxin.

2. Purification of λ-enzyme led to parallel purification of λ-antigen, and the two are considered to be identical.

3. λ-Enzyme was purified about 40 times in terms of the non-dialysable nitrogen in the crude filtrate from the casein medium in which it was already about 40 times as pure as in ordinary meat broth media.

4. The enzyme preparations attacked hide powder, gelatin, casein, haemoglobin and seracin, but had no effect on native collagen.

5. The optimum pH was pH 6·0–7·5.

6. The enzyme was destroyed at values below pH 5 or above 9 or by heating to 60° for 10 min.

7. Citrate inhibited the enzyme strongly, but there was no evidence for activation by calcium or magnesium ions. The enzyme was slightly inhibited by iodoacetic acid and cyanide and strongly inhibited by cysteine.

8. Its properties were compared with those of collagenase and the 'secondary' enzyme of *Clostridium welchii* Type A.

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Absorption and Excretion of the Mono-sodium Salt of Thyroxine Labelled with Radioactive Iodine

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There has been doubt in the past as to the efficacy of thyroxine administration by the oral route; some workers have even considered that thyroxine is inactive by mouth. A preference has been shown for the administration of dried glandular extracts, in which thyroxine is bound to protein or polypeptide. It was believed that thyroxine, being a relatively insoluble substance, was poorly absorbed from the gastro-intestinal tract (Harington, 1933). However, Schittenhelm & Eisler (1932) and Barnes & Bueno (1933), using Thiry-Vella fistulas in dogs, were able to show that thyroxine was absorbed from the closed intestinal loops. There is some evidence that the form in which thyroxine is administered has a bearing on its activity after oral administration, and Thompson, Thompson & Dickie (1933) found that thyroxine by this route was most active as the sodium salt and that it can be used successfully in the treatment of myxoedema (cf. also Hart & Maclagan, 1950). Bailey, Bartlett & Folley (1949) have recently shown that sodium L-thyroxine by mouth also stimulates milk secretion in lactating cows.

Following the development of an improved synthesis of thyroxine (Borrows, Clayton & Hems, 1949; Clayton & Hems, 1950) it became of interest to obtain data on its absorption after oral dosage. We have prepared and administered the mono-sodium salt of DL-thyroxine, labelled with radioactive ^{131}I in the 3′- and 5′-positions, to rats and cats in small doses with a view to determining its distribution and excretion.

The earlier work on the fate of thyroxine in the body has been reviewed by Elmer (1938) and Leblond (1948). In most of the earlier experiments the distribution of thyroxine was studied either by chemical estimation of iodine or by biological assays which, in view of low tissue concentrations and lack of specificity, were far from satisfactory. Such studies have been considerably facilitated by the advent of labelled thyroxine, which was first prepared by Joliot, Courrier, Horeau & Süe (1944*a*; see also Horeau & Süe, 1945). These investigators carried out a few experiments on rabbits (Joliot *et al.* 1944*a*, *b*; Joliot, 1945). The first detailed studies are due, however, to Gross & Leblond (1947), who gave relatively large intravenous injections of labelled thyroxine to rats.

We have administered physiological doses by the oral, subcutaneous and intravenous routes to both rats and cats. A preliminary account of our results has been published (Clayton, Free, Page, Somers & Woollett, 1949).

EXPERIMENTAL

Preparation of labelled thyroxine

The labelled thyroxine was prepared from 3:5-diiodothyronine by a modification of the method of Clayton & Hems (1950). To a solution of ^{131}I (1 mc.) as iodide in buffered bisulphite solution (pH 7; approx. 1 ml.), 2·3 mg. of NaI were added. The solution, in a centrifuge tube, was overlayered with 1 ml. of ether, and one drop of N-HCl and excess 0·3 % H_2O_2 solution (approx. 0·5 ml.) were added. After occasional shaking for 2 hr., the lower iodine-free layer was removed by means of a pipette. DL-3:5-Diiodothyronine (2 mg.) in 0·06 ml. of 33 % (w/v) aqueous ethylamine solution was added and the mixture shaken carefully when the iodine colour was discharged. The ether layer was removed by blowing a stream of air over the surface, and the aqueous layer was diluted with 1 ml. of water and adjusted to between pH 4 and 5 by the addition of glacial acetic acid. The precipitated thyroxine was spun down in a centrifuge and washed with successive amounts (1 ml.) of distilled water until the washings contained less than 5 % of non-thyroxine ^{131}I as demonstrated by *n*-butanol extraction of the aqueous washings (Blau, 1935). About 14 washings were

required. The thyroxine (about 2 mg.) was taken up in 10 ml. of 0·02 N-Na_2CO_3 solution and assayed for thyroxine polarographically (cf. Borrows, Hems & Page, 1949). A 1 ml. portion of the solution was added to a mixture of 0·125 ml. of 2·0 N-Na_2CO_3, 1·0 ml. of redistilled *iso*propanol and 0·5 ml. of 10 % (w/v) tetramethylammonium bromide. The mixture was made up to 5·0 ml. with distilled water and transferred to the cell of a recording polarograph (Cambridge Instrument Co.). After passing N_2 for 10 min., the sample was examined over the potential range −0·6 to −1·8 V. The heights of the first step and of the total steps were measured. Typical polarograms for thyroxine and diiodothyronine in a 20 % (v/v) *iso*propanolic solution containing 1·0 % (w/v) of tetramethylammonium bromide and 2·65 % (w/v) Na_2CO_3 are shown in Fig. 1. Thyroxine gives three polarographic steps, the

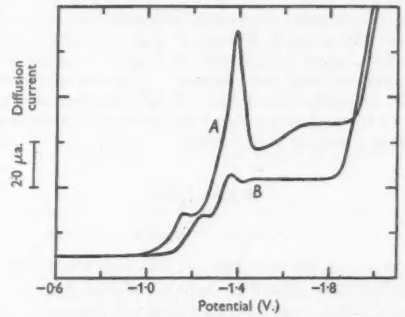

Fig. 1. Polarograms for $6·0 \times 10^{-4}$ M-thyroxine (*A*) and $6·0 \times 10^{-4}$ M-diiodothyronine (*B*) in 20 % (v/v) *iso*propanolic solution containing 1·0 % (w/v) of tetramethylammonium bromide and 2·65 % (w/v) of Na_2CO_3. The dropping mercury electrode at a pressure of 55·5 cm. of mercury and on open circuit in 0·1 N-KCl at 25° had a drop time of 3·13 sec; the weight of Hg dropping per sec. was 1·82 mg.

first step being at −1·12 V. (measured against the saturated calomel electrode); and diiodothyronine two steps, the first step being at −1·18 V. It is thus not possible to determine thyroxine directly in the presence of diiodothyronine. However, the total height of the three thyroxine steps is 3·4 times that of the first thyroxine step, whereas the total height of the two diiodothyronine steps is only twice that of the first diiodothyronine step. A value of 3·4 for the ratio of the total step height to that of the first step will therefore indicate that the labelled sample contains at least 90 % of thyroxine; any impurity is due to unchanged and unlabelled diiodothyronine. In these circumstances the height of the first step gives a measure of the amount of labelled thyroxine in the solution.

The specific activity of the labelled thyroxine was about 100 μc./mg.

In order to reduce the possibility of exchange between ^{131}I and ^{127}I, all labelled thyroxine solutions and extracts were kept at alkaline pH. Miller, Anderson, Madison & Salley (1944), and Frieden, Lipsett & Winzler (1948), have shown, and we have confirmed this observation, that exchange between iodide and either 3:5-diiodotyrosine or thyroxine proceeds rapidly at pH 5·0. Exchange was negligible at alkaline pH.

Biological activity of the labelled thyroxine

The biological activity of the material by the oral route was compared with a standard reference sample of pure sodium DL-thyroxine, using the method of Smith, Emmens & Parkes (1947) for determining thyroid activity. The method depends on the lowering of survival times of mice enclosed in jars after administration of thyroxine. Mice weighing approximately 20 g. were divided into two groups of 20. In one group every animal received 20 μg. of ordinary Na DL-thyroxine, in the other 20 μg. of labelled Na DL-thyroxine. The thyroxine was dissolved in 0·02 N-Na_2CO_3 and given orally on alternate days for 1 week, so that each animal received in all 80 μg. A control group of 20 mice received Na_2CO_3 solution alone. The mean survival times are shown in Table 1. The results show that both thyroxine solutions were biologically active and that the activity of labelled thyroxine was not significantly different from that of the control.

Table 1. *Survival times of mice after treatment with labelled or normal thyroxine*

Group	Mean survival time (min.)	Standard error (min.)
Controls	140	±6·8
Non-labelled monosodium thyroxine	96·6	±3·9
Labelled monosodium thyroxine	104·5	±3·5

Technique of animal experiments

Rat experiments. In our initial experiments, we examined the excretion and breakdown of labelled thyroxine by rats. Wistar strain rats of the same age and weight (70–100 g.) were placed in metabolism cages in which the urine and faeces could be collected separately. Throughout the experiments, the rats received a stock diet consisting of a paste prepared from whole-meal flour, dried milk and dried yeast, mixed with water. Paired rats in each cage were given single oral or subcutaneous doses of 1 mg. of labelled thyroxine/kg. of body weight, either as a solution of the monosodium salt in 0·02 N-Na_2CO_3 or as a suspension. The thyroxine suspensions were prepared by the careful addition of 0·1 N-HCl to a Na_2CO_3 solution, the pH being adjusted to 4·5. The urine and faeces were collected every 24 hr. for a period of 4 days and examined for total radioactive iodine, labelled thyroxine, labelled 3:5-diiodotyrosine and labelled iodide. At the end of each collection period, the metabolism cages, funnels and separators were washed down with a 'carrier' solution consisting of 0·4 N-NaOH containing 0·01 % of DL-thyroxine, 0·01 % of DL-3:5-diiodotyrosine and 0·01 % of NaI. The final washings were carried out with distilled water. All the washings were added to the urine samples, the total volumes being recorded. At the end of the experiment the rats were killed and the thyroids, liver, kidneys and carcasses examined for radioactive iodine. The experiment was repeated on a second group of rats.

Cat experiments. A series of cat experiments were undertaken to determine the proportion of ^{131}I appearing in the bile. Since it was necessary to use anaesthetized cats the duration of these experiments was comparatively short. After anaesthetization with ether, followed by tracheotomy, the femoral vein was exposed and cannulated. A chloralose

solution (0·08 g./kg. of body weight) was then introduced intravenously during the remainder of the anaesthesia. For collection of the urine, the abdomen was opened and the urethra cannulated, thus enabling the bladder to be completely emptied at each collection and so giving representative samples. A continuous collection of bile was obtained by cannulation of the common bile duct, the gall bladder being isolated by ligation of the cystic duct. Samples of blood were obtained at hourly intervals from the carotid artery.

Labelled thyroxine in 0·02 N-Na_2CO_3 was administered to the cats intraduodenally, subcutaneously or intravenously, the doses being from 34 to 100 μg. of thyroxine/kg. of body weight. The solutions were injected directly into the duodenal lumen, since preliminary experiments had shown that there was a considerable delay in the emptying of the stomach in anaesthetized animals, most of the dose being still present in the stomach at the end of the experiment. Three cats were treated by each route. Samples of blood, urine and bile were collected every hour for a period of 6 hr. and examined for total radioactive iodine, labelled thyroxine, labelled 3:5-diiodotyrosine and labelled iodide. At the end of the experiments the cats were killed and the thyroids, liver, stomach, kidneys and the small and large intestines removed and examined for radioactive iodine.

Analytical technique

Urine and blood. Samples of cat blood (10 ml.) and urine (diluted to 10 ml.) containing radioactive iodine were digested on a steam bath for 4 hr. with 2 ml. of 2 N-NaOH containing as 'carrier' 0·01 % thyroxine, 0·01 % 3:5-diiodotyrosine, and 0·01 % NaI. The pH of the digest was adjusted with 35 % (v/v) nitric acid to about 9, and a 10 ml. portion transferred to a jacketed Geiger-Müller counter (cf. Veall, 1948). The number of disintegrations per minute was measured on a Dynatron scaling unit. The background count was about 10/min.

Faeces. Samples of faeces (5 g.) were digested on a steam bath for 4 hr. with 20 ml. of 2 N-NaOH containing as 'carrier' 0·01 % of thyroxine, 0·01 % of 3:5-diiodotyrosine and 0·01 % of NaI. The residual solid matter was removed by centrifuging and digested for 4 hr. with a further 10 ml. of 2 N-NaOH. A third extraction was performed and the pH of the combined alkaline extracts adjusted with 35 % (v/v) nitric acid to 9·0. A 10 ml. portion was examined in the jacketed Geiger-Müller counter.

Stomach, intestines, liver, thyroid and other organs. In our earlier experiments the organs were digested with 2 N-NaOH; as there were difficulties due to gel formation, Howarth's (1949) technique was subsequently adopted. The organ was minced and a sample portion (5 g.) refluxed for 4 hr. with 10 ml. 2 N-LiOH in 20 % (v/v) ethanol, containing as 'carrier' 0·01 % thyroxine, 0·01 % 3:5-diiodotyrosine and 0·01 % NaI. The residual solid matter was centrifuged off and the supernatant liquid, after adjustment to pH 9, examined in the counter.

Labelled thyroxine and iodide determinations. The labelled thyroxine in the extracts was isolated by the method of Blau (1935). The sample was acidified (pH 3·5) and extracted successively with an equal volume and a half volume of *n*-butanol. The combined butanol extracts were then washed successively with an equal and a half volume of 4 N-NaOH containing 5 g. Na_2CO_3/100 ml. The butanol extract was examined; after due allowance for the specific gravity of butanol (cf. Veall, 1948) the count gave a measure of the labelled thyroxine in the sample.

The radioactivity in the combined aqueous layers was identified as iodide by the method of Gross & Leblond (1947). Inactive NaI (100 mg.) was added to the aqueous layer, which was then neutralized with conc. HNO_3, the iodide being precipitated by a solution of 10 % (w/v) $AgNO_3$ in 2 % (v/v) HNO_3. The precipitate was centrifuged off, redissolved in 10 ml. of hot 25 % (w/v) $Na_2S_2O_3$ and examined. The supernatant liquid from the $AgNO_3$ precipitation gave a measure of the labelled 3:5-diiodotyrosine in the sample.

Radio autographs. Several samples of urine and faeces containing large quantities of radioactive iodine were examined by the radio autograph technique of Fink, Dent & Fink (1947). A sample of a neutral extract was run on a phenol one-dimensional paper chromatogram, and a radio autograph obtained by placing the paper chromatogram in contact with a Kodak 'Industrex' type D X-ray film for up to 7 days. After development of the film, exposed areas appeared, owing to the presence on the chromatogram of 'spots' containing radioactive iodine. It was confirmed that in urine the radioactive iodine was present as iodide and in faeces as a mixture of thyroxine and iodide.

RESULTS

Rat experiments

In our initial experiments we followed the excretion of radioactive iodine by rats for a period of 4 days after a single oral or subcutaneous doses of either a solution or a suspension of labelled thyroxine. The subsequent excretion and final distribution of ^{131}I are shown in Tables 2 and 3. The results are the mean values obtained from two separate experiments and indicate that the solution and the suspension are metabolized in the same way. The amounts of labelled diiodotyrosine found were negligible and have hence been omitted from the Tables. The results show clearly that after oral administration, absorption occurred from the intestinal tract. There was a higher percentage (65 and 57 %) of ^{131}I in the faeces of the orally treated rats than in those of the injected rats (44 and 39 %). By both routes from 50 to 80 % of the ^{131}I administered had been excreted in the first 24 hr. and, allowing for experimental errors, almost all the dose had been excreted by the fourth day. The ^{131}I was present in the urine almost, if not entirely, as iodide, but in the faeces nearly three-quarters of the ^{131}I present was butanol-soluble (Tables 2 and 3). This was confirmed by the radio autographs. Analysis of the individual organs and the carcasses of the rats at the end of the experiments showed very little residual ^{131}I in the animals. Only a very small amount of ^{131}I was present in the liver and kidneys; by far the highest concentrations were found in the thyroid glands (1·4–4·8 % of the dose). Only a small proportion of the ^{131}I in the thyroids was butanol-soluble (Table 3).

Table 2. *Excretion of* ^{131}I *by groups of two rats after dosage with* 1·0 *mg. of labelled thyroxine per kg. of body weight*

		^{131}I as percentage of ^{131}I in dose							
		Group A, oral dose of solution containing 192 μg. of labelled thyroxine		Group B, subcutaneous injection of solution containing 187 μg. of labelled thyroxine		Group C, oral dose of suspension containing 180 μg. of labelled thyroxine		Group D, subcutaneous injection of suspension containing 173 μg. of labelled thyroxine	
Sample	Time of collection (hr.)	Total ^{131}I	Thyroxine ^{131}I	Total ^{131}I	Thyroxine ^{131}I	Total ^{131}I	Thyroxine ^{131}I	Total ^{131}I	Thyroxine ^{131}I
Urine	0–24	26·8	0·6	36·2	0·2	26·2	0·5	34·1	0·3
	24–48	5·1	0·2	9·1	0·1	5·7	0·2	6·8	0·2
	48–72	2·1	0·1	2·1	0·1	1·2	0·1	3·1	0·1
	72–96	0·6	0·1	0·9	0·1	0·5	0	1·2	0·1
Faeces	0–24	55·8	35·0	24·8	15·0	48·2	36·5	19·1	13·2
	24–48	8·1	5·2	15·3	13·2	6·9	5·1	14·2	10·2
	48–72	1·4	1·1	3·2	2·6	1·5	1·4	4·2	4·6
	72–96	0·2	0·1	1·1	0·6	0·1	0·1	0·8	0·8

Table 3. *Distribution of* ^{131}I *in groups of two rats four days after dosage with* 1·0 *mg. of labelled thyroxine per kg. body weight*

(The total ^{131}I content of the liver, kidney and 'rest of body' samples was too low to permit an accurate thyroxine ^{131}I determination.)

	^{131}I as percentage of ^{131}I in dose							
	Group A, oral dose of solution containing 192 μg. of labelled thyroxine		Group B, subcutaneous injection of solution containing 187 μg. of labelled thyroxine		Group C, oral dose of suspension containing 180 μg. of labelled thyroxine		Group D, subcutaneous injection of suspension containing 173 μg. of labelled thyroxine	
Organ	Total ^{131}I	Thyroxine ^{131}I	Total ^{131}I	Thyroxine ^{131}I	Total ^{131}I	Thyroxine ^{131}I	Total ^{131}I	Thyroxine ^{131}I
Thyroid	3·9	0·6	3·7	0·5	1·4	0·2	4·8	0·9
Liver	<0·03	—	<0·03	—	0·06	—	0·05	—
Kidney	0·006	—	<0·003	—	<0·003	—	<0·003	—
Rest of body	0·8	—	1·5	—	0·5	—	0·55	—
Urine: total	35	1·0	48	0·4	34	0·8	45	0·7
Faeces: total	65	41	44	31	57	43	39	29

Cat experiments

The previous experiments showed that following the oral administration of thyroxine to rats absorption occurred. However, interpretation of the results is complicated by the secretion of thyroxine into the intestinal lumen. It is known that thyroxine is secreted in the bile (Gross & Leblond, 1947). It was therefore of interest to carry out experiments on anaesthetized cats in which the common bile duct can be cannulated, thus enabling us to determine the rate and form of the ^{131}I excretion. Re-absorption of ^{131}I from the intestinal lumen would also be prevented. Anaesthetized cats were therefore injected with labelled thyroxine subcutaneously, intravenously or directly into the duodenal lumen. Because of the large number of extractions involved, it was only possible to study three cats by each route. In general, the dose administered was equivalent to 34 μg. of thyroxine/kg. of body weight, but occasionally with less active material it was necessary to employ 100 μg./kg. The experimental results, when calculated as a percentage of the dose administered, were of the same order at both dose levels; we have therefore given the mean results of three experiments by each route in Tables 4 and 5. The percentage of the dose present in the blood was calculated on the assumption that the blood volume in millilitres was equivalent to 6·5% of the body weight in grams (cf. Dukes, 1947).

Fifteen minutes after injecting labelled thyroxine into the intestinal lumen, radioactive iodine was detected in the blood, indicating that some absorption had occurred. Radioactive iodine was found in the bile after all three methods of administration, and there appeared to be a direct relationship between the bile and blood concentrations of ^{131}I. Of the total ^{131}I in the bile (cf. Table 6) about 50 % was present as unchanged thyroxine, except after intravenous administration, when the proportion of thyroxine ^{131}I showed a tendency to fall to about 30 % of the total after the third hour.

Table 4. *Metabolism of* ^{131}I *by cats after dosage with labelled thyroxine*

Method of administration and quantity administered	Time of collection (hr.)	Percentage of ^{131}I in dose in blood*	Percentage of ^{131}I in dose secreted in bile	Percentage of ^{131}I in dose excreted in urine
Intraduodenal injection (34 μg. of labelled thyroxine/kg.)	0–1	1·4	0·05	0·05
	1–2	1·6	0·16	0·07
	2–3	2·7	0·19	0·16
	3–4	2·8	0·18	0·24
	4–5	3·1	0·20	0·34
	5–6	2·9	0·22	0·80
Subcutaneous injection (100 μg. of labelled thyroxine/kg.)	0–1	3·1	0·23	0·23
	1–2	3·3	0·47	0·26
	2–3	3·6	0·66	0·40
	3–4	4·4	0·49	0·48
	4–5	4·6	0·45	0·97
	5–6	4·9	0·31	0·86
Intravenous injection (34 μg. of labelled thyroxine/kg.)	0–1	18·7	2·25	0·80
	1–2	13·5	1·82	0·54
	2–3	12·0	1·25	0·83
	3–4	9·2	0·70	1·2
	4–5	7·8	0·46	1·3
	5–6	5·8	0·50	2·0

* Calculated on the assumption that the blood volume in ml. was equivalent to 6·5 % of the animal's body weight in g.

Table 5. *Distribution of* ^{131}I *in organs of a cat 6 hr. after dosage with labelled thyroxine*

(— means 'not determined')

Organ or fluid	^{131}I found in organ or fluid as percentage of ^{131}I administered: Intraduodenal injection of 34 μg. of labelled thyroxine/kg.	Subcutaneous injection of 100 μg. of labelled thyroxine /kg.	Intravenous injection of 34 μg. of labelled thyroxine/kg.
Total urine (0–6 hr.)	1·7	3·2	6·7
Total bile (0–6 hr.)	1·0	2·6	7·0
Blood	2·9	4·9	5·8
Stomach	4·2	2·8	8·8
Small intestine and contents	74	1·7	4·6
Large intestine and contents	0·57	0·36	0·57
Thyroid gland	0·05	0·002	0·10
Liver	1·8	4·3	6·0
Kidney	0·72	1·75	3·3
Adrenal gland	<0·003	—	0·024

Table 6. *Distribution of labelled thyroxine in bile of a cat which received a single dose of labelled thyroxine*

(— means 'not determined')

Time after dosage (hr.)	^{131}I present as thyroxine expressed as percentage of total ^{131}I in bile: Intraduodenal injection of 34 μg. of labelled thyroxine/kg.	Subcutaneous injection of 100 μg. of labelled thyroxine/kg.	Intravenous injection of 34 μg. of labelled thyroxine/kg.
0–1	—	—	40
1–2	50	54	45
2–3	48	47	48
3–4	48	39	28
4–5	—	37	34
5–6	51	—	40

The ^{131}I excreted in the urine was almost entirely present as iodide. It was of interest to observe that the urine concentrations had hardly reached a maximum by the sixth hour. The blood levels indicate that, following intravenous injection, excess thyroxine is quickly removed from the circulation, the ^{131}I content rapidly falling from its initial high level. By the subcutaneous and intraduodenal routes, after an initial sharp rise during the first 1–2 hr., the ^{131}I concentration levels off and is maintained more or less constant during the remaining period of the experiment. Probably after administration by these two routes an equilibrium becomes established between absorption and excretion.

At the end of the experiments the ^{131}I remaining in the body was chiefly concentrated in the liver,

kidneys and gastro-intestinal tract. A high concentration was also found in the stomach, even in the injected animals. In one experiment 11·6 % of the original intravenous dose was recovered from the stomach, the ^{131}I being present mainly as iodide. After intraduodenal administration about 75 % of the initial dose was still unabsorbed at the end of 6 hr., indicating that the rate of absorption was not so rapid as the rat experiments would suggest, though it is possible that the anaesthetic had some influence. Very little radioactivity was found in the thyroids of these animals, except in one experiment, the results of which were discarded because the original labelled thyroxine solution had deteriorated and contained some labelled iodide.

DISCUSSION

The presence of radioactivity in the blood and of labelled thyroxine in the bile after oral administration confirmed that absorption of the monosodium salt does occur. However, the rapid disappearance of thyroxine from the blood stream precludes the use of blood levels for measuring the efficiency of the absorption. Labelled thyroxine was found in the faeces of the injected rats, as has been reported by Gross & Leblond (1947). It was confirmed in our experiments, using cats with cannulated bile ducts, that the faecal excretion of thyroxine was due to its elimination in the bile. Therefore, thyroxine present in the faeces of the orally treated rats could not be entirely due to failure of absorption; interpretation of the results is further complicated by the fact that some at least of the thyroxine and its metabolites excreted in the bile would be re-absorbed from the intestinal lumen (cf. Schittenhelm & Eisler, 1932; Gross & Leblond, 1947). Our results indicate that about 70 % of an oral dose of sodium DL-thyroxine is absorbed by rats. Frieden, Tuckich & Winzler (1949) found by the goitre-prevention test that sodium DL-thyroxine exhibited half of its parenteral activity when administered orally to rats. These results are in contrast with the suggestions of Bailey *et al.* (1949) that only 6·3 % of an oral dose of sodium L-thyroxine was absorbed by cows and of Turner & Reineke (1946) that 12·5 % of an oral dose of sodium DL-thyroxine was absorbed by sheep, but they compare favourably with the observation of Monroe & Turner (1949) who found that 45 % of an oral dose of sodium DL-thyroxine was absorbed by the domestic fowl. The variations may well be due to species differences.

In general, our results on the excretion and distribution of thyroxine are in close accord with those of Gross & Leblond (1947), in spite of the fact that these workers used higher intravenous doses. It would appear that the administration of physiological doses of thyroxine raises the concentration of the circulating hormone above normal levels, and that the body then inactivates the excess by direct excretory and destructive mechanisms.

Previous authors have reported that thyroxine tends to concentrate in the liver (Krayer, 1928; Gross & Leblond, 1947), where it is partially broken down and then eliminated through the bile (Kendall, 1919, Asimoff & Estrin, 1931; Schittenhelm & Eisler, 1932; Barnes, 1933; Barnes & Chang, 1933; Elmer & Luczynski, 1933, 1934). Our experiments with cats (cf. Table 6) showed that about 50 % of the thyroxine in the bile had been broken down with formation of inorganic iodide, observations that are in reasonable agreement with those of Elmer & Luczynski (1933, 1934), who injected larger doses (2–6 mg.) of thyroxine intravenously and into the stomach of rabbits. About 5·0–6·5 % of the iodine from the intravenous dose and 0·8–1·4 % from the stomach dose appeared in the bile during the first 12 hr. After the intravenous dose, Elmer & Luczynski found that about 50 % of the iodine in the bile was present as thyroxine and after the stomach dose about 20 %. The results listed in Table 4 suggest that there is a relationship between the blood and bile concentrations of radioactive iodine.

Very little of the administered thyroxine was retained in the body, the rats excreting between 50 and 80 % of the dose within the first 24 hr. and practically all by the end of the fourth day. It is difficult to explain these results in view of the known delay in the onset of maximal metabolic effects.

Thyroxine itself was never found in significant quantities in the urine of cats or rats. Urinary excretion of ^{131}I was entirely as iodide (cf. Asimov & Estrin, 1931; Elmer & Scheps, 1933, 1934; Gross & Leblond, 1947); the kidney does not appear to be permeable to thyroxine itself. Further evidence that the kidney deals only with thyroxine decomposition products is indicated by the delayed excretion of ^{131}I shown in the cat experiments.

Studies on the final distribution of ^{131}I in the body showed differences depending on the duration of the experiments. In the short-term cat experiments the highest proportions of radioactive iodine were found in the liver, kidneys and gastro-intestinal tract, with very little in the thyroid glands. At the conclusion of the rat experiments, which extended over 4 days, practically all residual radioactive iodine was concentrated in the thyroid glands and about 85 % was present as iodide. It is therefore fairly certain that the thyroid gland is incapable of absorbing and storing its own hormone. The gland probably absorbs the inorganic iodide liberated from the decomposed thyroxine and incorporates it in new thyroxine. Taurog, Tong & Chaikoff (1949) have shown that 24 hr. after an injection of labelled iodine only 15 % of the labelled iodine in the thyroid was present as thyroxine.

After this paper had been prepared for publication, we received a copy of Leblond's (1949) article on the distribution of ^{131}I after intravenous administration of physiological doses of labelled thyroxine for rats. The labelled thyroxine had been prepared by the butanol extraction of the thyroxine fractions from the thyroids of rats previously treated with radioactive iodine. The distribution of radioactive iodine in two rats 2 hr. after dosage was studied. Our results are in general agreement with those of Leblond (1949).

SUMMARY

1. Single doses of the monosodium salt of DL-thyroxine labelled with radioactive iodine in the 3′- and 5′-positions were administered either in solution or as a suspension, orally or subcutaneously, to rats; the subsequent excretion in urine and faeces was determined over a 4-day period. The final distribution of radioactive iodine in the carcass was studied.

2. It was shown that the thyroxine in solution and as a suspension is metabolized in the same way and that it is absorbed from the gastro-intestinal tract. The radioactive iodine in the urine was present almost entirely as iodide, whereas that in the faeces was present in both organic and inorganic forms.

3. Single physiological doses of labelled monosodium DL-thyroxine were administered intraduodenally, subcutaneously, or intravenously to anaesthetized cats; the subsequent radioactive iodine blood levels and urinary and biliary excretion rates were measured over a 6 hr. period. The distribution of labelled thyroxine and its breakdown products was studied.

4. For all routes of administration, the amount of radioactive iodine in the bile was related to the blood concentrations; that in the bile was present in approximately equal proportions as thyroxine and iodide. Appreciable amounts of iodine were secreted into the stomach.

5. Rat thyroids, in contrast to cat thyroids, contained appreciable quantities of radioactive iodine; very little of the radioactive iodine in the rat thyroids was present as thyroxine.

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A New Apparatus for the Determination of Carbon in Biological Materials by Wet Combustion

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In the course of studies upon the metabolism of carbon sources used in media for the growth of mould fungi (Hockenhull, 1946), a method of analysis for carbon by wet combustion due to McCready & Hassid (1942) was modified so that the evolved carbon dioxide could be collected in baryta in a special spiral bubbler after Pearce & Robertson (1936). This apparatus, while giving satisfactory results for quantities of carbon up to 10 mg., had the following disadvantages: (i) neoprene tubing (being more resistant to acid attack than rubber) had to be used for connecting the condenser and the three bubblers used; (ii) the normal type bubblers used for scrubbing (cf. *E* and *F* on Fig. 1) were awkward to refill; (iii) the apparatus took up too much horizontal space; (iv) although the spiral bubbler was potentially highly efficient, individual ones varied greatly in their performance. Fig. 1 illustrates a modified apparatus, made entirely in Pyrex glass.

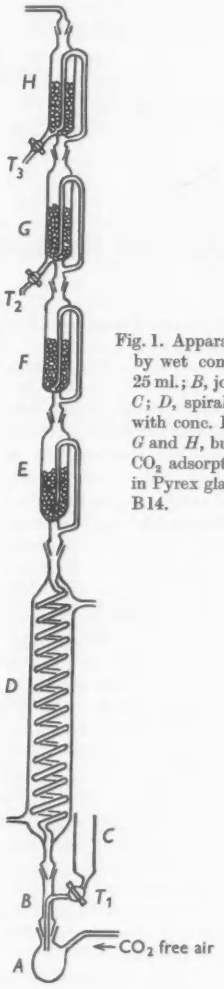

Fig. 1. Apparatus for determination of carbon by wet combustion: *A*, combustion flask 25 ml.; *B*, jointed tube carrying tap funnel, *C*; *D*, spiral water condenser; *E*, bubbler with conc. H_2SO_4; *F*, bubbler with water; *G* and *H*, bubblers with standard alkali for CO_2 adsorption. T_1, T_2 and T_3, taps. All in Pyrex glass; standard ground joints, all B14.

Method of operation

Preparation of the apparatus. The joints below *F* and tap T_1 were lubricated with syrupy phosphoric acid and the joints above *F* with water. Taps T_2 and T_3 were treated with vaseline or tap grease. All the bubblers contained beads (6–7 mm.). The bubbler *E* contained conc. H_2SO_4; *F*, water; *G*, 25 ml. of 0·1 N-$Ba(OH)_2$ and *H*, 5 ml. of 0·1 N-$Ba(OH)_2$ with water and beads to fill sufficiently. A little *sec.*-octanol may be added to *G* and *H* to prevent persistent foaming.

Method of analysis. The sample should contain about 10 mg. of C. It should be dry, or in not more than 0·2 ml. of aqueous solution, in which case about 200 mg. of KIO_3 must be added. It is placed in *A* (capacity 25 ml.) and the apparatus is connected up. A brisk stream of CO_2-free air (more than 100 ml./min.) is sucked through. Van Slyke-Folch combustion mixture (4 ml.) (McCready & Hassid, 1942) is added from *C*. *A* is heated so that the contents boil in 2 min., the flame being removed if evolution of I_2 begins. The reaction mixture is maintained at the boil, while 2·5–3·0 l. of CO_2-free air pass through the apparatus and carry the CO_2 formed into the alkali.

When the process has been completed, suction is stopped and the apparatus equilibrated with the atmosphere, taking care not to allow siphoning back of the contents of the bubblers.

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H and *G* are removed and their contents are run off at T_1 and T_2 into a covered flask. *H* and *G* are washed out twice with CO_2-free water and the washings added to the rest of the alkali. The residual $Ba(OH)_2$ is titrated with 0·1 N-HCl, using thymolphthalein as indicator. Each determination takes about 30 min.

More dilute alkali and HCl are used for smaller quantities of sample.

Accuracy of method

Five 0·5 ml. portions of sucrose solution, each containing 10·8 mg. of carbon, were dried at 90° before analysis. The results were 11·0, 10·9, 10·8, 11·3 and 10·6 mg. (mean 10·92 mg., S.D. 0·23 mg.). Five determinations of carbon content were carried out upon potassium hydrogen phthalate (A.R.) using approx. 10 mg. of dry solid, giving a mean of 47·0 % (S.D. 0·8 %, calculated 47·1 %). Five determinations were carried out without prior drying down upon 0·2 ml. of a solution containing approx. 10 mg./ml. of potassium hydrogen phthalate; the results gave a mean of 47·6 % (S.D. 1·3 %; calculated by direct titration 47·2 %). Determinations carried out on mould metabolism solutions, after drying down, and upon dried mycelial felts have given the same order of reproducibility at approx. 5 mg. carbon/sample (S.D. 2·1 and 1·9 % respectively).

SUMMARY

A new apparatus for the determination of carbon in biological materials by wet combination is described.

My thanks are due to Mr K. Bartholemew, glass-blower, for making the apparatus.

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Polarographic Determination of Lead in Urine

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Most of the methods that have been proposed in recent years for the determination of small amounts of lead are based on colorimetry and the use of dithizone (Tompsett & Anderson, 1935; Kerch, 1940). For microdeterminations of metals the methods of polarographic analysis are attractive since they are simultaneously qualitative and quantitative, and the diffusion currents from which values are obtained can be measured accurately and reproducibly. Since extraneous lead is the only substance which can contribute to the reagent blank, the latter is generally smaller than would be found in a colorimetric method, which may also be less specific.

Procedures for the polarographic determination of lead in urine have been described by Bombach & Cholak (1941), Reed & Gant (1942) and Weber (1947). Bomach & Cholak (1941) introduced a stage of electrolytic separation, and each of the three methods involved dry ashing at high temperatures; with the methods described below it has been possible to reduce the time taken for each determination, to avoid dry ashing, and to use less specialized equipment than is demanded by the above authors, while considerably improved accuracy and sensitivity are attained in a special modification.

Lead may be determined polarographically in a variety of conditions (Kolthoff & Lingane, 1941); when dissolved in acid citrate or tartrate solutions (Suchy, 1931; Shakhov, 1939) the conditions are especially suitable since the half-wave potential is smaller in acid than in neutral or alkaline solution. Since the lead 'wave' is found at a lower applied potential in acid media, there is a greater chance of any other electro-reducible substances giving their waves after lead, in which case their presence will not interfere. The author has found that the diffusion current of lead may be readily and accurately determined in 0·2 M-dipotassium hydrogen citrate (pH 5·2), and that in this medium the waves are not in any way distorted by the formation of maxima; the half-wave potential was found to be −0·49 V. (measured against the Standard Calomel Electrode).

In the present work, the lowest concentration of lead in the polarograph cell which gives a readily measurable diffusion current has been found to be of the order of 500 μg./l. Most values reported in the literature for the urinary excretion of lead by normal subjects are below 100 μg./l. (Kerch, 1940; Tompsett & Anderson, 1935); a process for concentration of the metal is therefore necessary. Also, since it is possible that urine may contain polarographically active substances which might interfere with the determination of lead, a partial isolation of the lead has been incorporated in the final method. This

twofold requirement is met by a double entrainment and a destructive oxidation of organic material; metals other than lead, and also inorganic anions, are not necessarily eliminated, but are found not to cause any inconvenience. The lead is obtained finally as a citrate complex dissolved in citrate buffer.

It has been shown that lead is entrained quantitatively in the precipitation of calcium either as oxalate at pH 4·5 (Ross & Lucas, 1935) or as phosphate in strongly ammoniacal solution (Ross & Lucas, 1933).

Although calcium forms a soluble complex citrate, calcium oxalate is not dissolved by solutions of either citric acid or citrates. Calcium phosphate precipitates, on the other hand, dissolve rapidly even in neutral citrate solutions. Since, however, substances other than calcium (e.g. magnesium and iron) are precipitated from urine by addition of ammonia, the phosphate precipitate obtained in ammoniacal solution is not always completely soluble in citrate buffer, so that with some samples of urine the maximum current for lead was found only after several hours; in one case the final value was 10 % greater than that found initially.

A rapid but rough method (method *A*) was therefore available. A precipitate of calcium phosphate was formed in urine, separated, washed, dissolved as completely as possible in citrate solution and the lead determined polarographically.

In a more accurate procedure (method *B*), calcium was precipitated as oxalate at pH 4·5, the solid was 'ashed' with perchloric acid and the calcium precipitated from alkaline solution as phosphate, which dissolved completely with the lead in citrate buffer. The second precipitation as phosphate was employed since it was difficult to buffer the ashed perchlorate solution reproducibly and with sufficient accuracy without increasing the volume to an undesirable extent.

Determination of, or allowance for, the reagent blank is as important here as in most micromethods. Therefore a compensation was introduced by treating two volumes (25 and 5 ml.) of urine with the same quantities of reagents, the lead content of the difference (20 ml.) being deduced by comparison of polarograms. Lead also forms a soluble complex in tartrate solutions, and it has been shown by Forche (1938) that adsorbed lead salts can be leached from insoluble material by tartrate solutions, although prolonged extraction (2 hr.) is required. Since, however, solutions of tartrates do not dissolve calcium oxalate or phosphate, a citrate medium was chosen, since, with this, complete solution of the solid material can be readily achieved, thus ensuring the presence of the adsorbed or co-precipitated lead in the liquid phase.

EXPERIMENTAL

Urine is collected as 24 hr. specimens in Winchester quart bottles which have been washed with warm HNO_3 (A.R., about 8M) and which contain 10 ml. of conc. HNO_3 (A.R. about 16M).

Reagents

These are all of A.R. quality.

Ammonia, sp.gr. 0·880.

Calcium chloride, 0·225M. The solution is freed from Pb by adding 0·005 vol. of the NH_4 oxalate solution (below), stirring from time to time for 1 hr. and then filtering through Whatman no. 541 paper.

Ammonium oxalate, 0·35M. The solution is adjusted to pH 4·5 with acetic acid and NH_3; it is freed from Pb by addition of 0·01 vol. $CaCl_2$ solution, standing, stirring and filtering as above.

Dilute oxalate solution, for washing precipitates, is prepared by diluting some of the above solution tenfold.

Perchloric acid, 60 % (as supplied by British Drug Houses Ltd.).

Phosphoric acid, 10 % (v/v) syrupy acid (1·9M).

Phosphate solution, for washing precipitates. 1 vol. of 0·067 M-Na phosphate buffer of pH 7·3 is diluted to 5 vol.

Citrate buffer, 0·2M-dipotassium hydrogen citrate, 21·61 g. $K_2C_6H_6O_7.H_2O$ and 7 g. $H_3C_6O_7.H_2O$ are dissolved together in water and made up to a total volume of 500 ml. This buffer is conveniently used in a burette; other reagents, excepting wash-liquids, are handled with Pasteur pipettes calibrated for the purpose.

Quartz chips are boiled in 8M-HNO_3 and rinsed with water.

The small (12 ml.) centrifuge tubes referred to below are of plain test tube form and are graduated at 2·0, 5·0 and 10·0 ml. Glassware is of Pyrex or other Pb-free glass and is rinsed with 8M-HNO_3 and with water.

Procedure

The urine, collected as described, has a pH between 1·2 and 2·2. A bulk sample is brought within the pH range 4·4–4·6 by the addition of NH_3. For 500 ml., about 3 ml. ammonia (sp.gr. 0·880) is required. The adjustment was usually made with the glass electrode, but bromcresol green was used externally in a few cases.

Method A. To 25 and 5 ml. of urine at pH 4·5 in 30 ml. centrifuge tubes are added 1 ml. 10 % H_3PO_4, 0·3 ml. $CaCl_2$ solution and 1 ml. NH_3 with intermediate mixing. After stirring at intervals for 20 min. the solids are centrifuged off, drained, washed into smaller tubes with the phosphate solution, respun, drained and dissolved in citrate buffer for polarographic determination as described for method *B*.

Method B. Duplicate samples of 25 ml. and also of 5 ml. are measured into 30 ml. centrifuge tubes, to each of which are then added 0·3 ml. of the $CaCl_2$ solution and 2 ml. of the NH_4 oxalate solution with agitation after each treatment. After 20 min., during which period the suspensions are shaken or stirred every 5 min., they are centrifuged (5 min. at 3000 rev./min.), and drained by inversion for 2–3 min. on filter paper. The precipitates are transferred to smaller tubes (12 ml.) by careful washing with a total volume of 10 ml. of the dilute NH_4 oxalate solution in successive small quantities with the aid of a glass rod. Solid material is spun off and drained as before. To each tube is now added 0·4 ml. $HClO_4$ and a quartz chip; oxidation is carried out for 15 min. on a hot

plate of temperature such that the solutions are clear and refluxing 3 cm. from the base of the tube after 5 min., with no further frothing due to decomposition of oxalate. When cool, the digests are each diluted with 5 ml. water, added so as to wash down the sides of the tubes; 1 ml. 10 % H_3PO_4 and 1 ml. NH_3 are run in with mixing, and water is added to a final volume of 10 ml. Ten minutes are allowed with two or three agitations before the deposit of Ca salts is spun down, drained, washed with 10 ml. of the phosphate solution, respun and drained. The solid is then dissolved in citrate buffer and made up to a definite volume (2 ml.) with the buffer. The precipitates dissolve readily on shaking. Generally, addition of 1·65 ml. buffer is required, but extraordinary concentrations of urinary Ca will vitiate this working rule.

Tubes containing the final solutions are placed in the 25° thermostat of the polarograph and O_2 is removed by passing a gentle stream of N_2 for 4–5 min. An assembly comprising the dropping electrode and a flexible bridge filled with saturated aqueous KCl is lowered into the solution, the N_2 tube is drawn above the surface and the polarogram is recorded over the range of applied potential −0·3 to −0·6 V. with suitable counter current; all potentials are measured against the Standard Calomel Electrode (S.C.E.). Sensitivity should be of the order of 10 cm. galvanometer deflexion for 1·0 μa. in the cell. The half-wave potential of Pb in these conditions has been found by the author to be −0·49 V. The differences between the heights of the lead 'waves' in the solutions derived from 25 and 5 ml. samples of urine represent values, corrected for the reagent blank, of the Pb originally present in 20 ml. urine. From calibration experiments this derived current can be interpreted in terms of μg. Pb/l. and hence per 24 hr. excretion.

A modification of method *B* was evolved for use with urine containing less than 50 μg. Pb/l. This is described after the account of the results obtained with larger amounts of Pb using methods *A* or *B*.

Calibration

For clinical purposes it is preferable to analyse samples of urine at pH 4·5 to which Pb has been added to give known

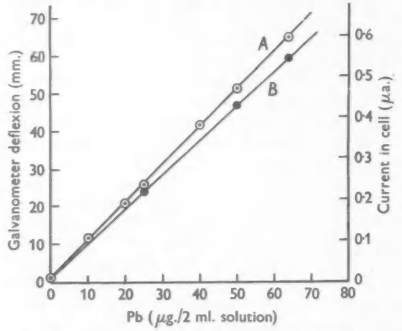

Fig. 1. Diffusion current of lead as a function of concentration. Curve *A*, Pb in 0·2 M-dipotassium hydrogen citrate; Curve *B*, as (*A*) in presence of Ca (approx. 0·03 M).

concentrations in the range 100–1800 μg./l. Thus a curve can be constructed from which concentrations of Pb in μg./l. can be read off directly against the difference in currents derived from 25 and 5 ml. samples. In the present work, however, curves were built up (Fig. 1) showing current as a function of concentration in the final solution with Pb (as nitrate) both in pure 0·2 M-citrate buffer and in a citrate solution in which Ca phosphate had been dissolved. This latter solution was made up by applying method *A* on a tenfold scale to 50 ml. water in place of urine.

Polarographic data

The instrument used is of the non-recording type, designed and constructed by the author. An enclosed galvanometer (Cambridge Spot, 450 Ω) is incorporated; damping is by condenser only with the sensitivity ($\frac{2}{3}$) employed in the present work; 1 μa. in the cell gives a deflexion of 10·9 cm. A saturated calomel electrode is used for reference, connected to the cell by means of a flexible bridge, with sintered-glass ends, which is filled with saturated KCl solution.

The characteristics of the dropping-Hg electrode were recorded as nearly as possible under working conditions; the electrode and salt-bridge were immersed in air-free 0·2 M-citrate buffer maintained at 25° while a potential of −0·5 V. (S.C.E.) was applied. The mean mass in mg. of Hg falling /sec. (m) from the capillary was found by collecting and weighing the Hg falling in a period of 1000 sec. Simultaneously, the time interval between the falling of successive drops (t sec.) was measured. These determinations gave $t = 2{\cdot}82$ sec; $m = 1{\cdot}388$ mg./sec., with a hydrostatic pressure at the capillary tip, maintained throughout the present work, of 45 cm. Hg. The 'capillary constant', $(m^{\frac{2}{3}}t^{\frac{1}{6}})$ therefore has the value 1·479 mg.$^{\frac{2}{3}}$ sec.$^{-\frac{1}{2}}$. The maximum excursion of the galvanometer in the analytical procedure was ±0·2 mm. The N_2 used for displacement of air from the solutions to be polarographed was taken from an industrial cylinder and was scrubbed with KOH-pyrogallol and with water.

The diffusion coefficient of Pb complex ion, calculated from the diffusion currents by the Ilkovic equation is $4{\cdot}5 \times 10^{-6}$ cm.^{2} sec.$^{-1}$ in pure 0·2 M-dipotassium hydrogen citrate. The limiting current was found to have a temperature coefficient of +2·6 %/degree in the range 18–28°. In the more complex medium made up to simulate that found in practice, which contains much Ca in solution, together with small amounts of phosphate, Na and traces of NH_3, the currents given by Pb are 91 % of the corresponding currents in pure citrate, i.e. the diffusion coefficient is now $3{\cdot}7 \times 10^{-6}$ cm.^{2} sec.$^{-1}$.

RESULTS

Recovery is almost quantitative over the range 100–1800 μg. lead/l. as shown in Table 1, where the results of current-difference interpretation made by reference to curve *B* of Fig. 1 are set out.

The consistency of the methods is illustrated in Table 2, where the results of duplicate estimations are recorded.

Five investigations into four cases of suspected lead intoxication have been made. In two of the cases, abnormally large amounts of urinary lead were found. Urine was collected over three consecutive periods of varying duration (Table 3); in the central period a course of 2:3-dimercaptopropanol (BAL) injections (individual doses of 1·5 mg./kg.

Table 1. *Recoveries of lead added to stocks of urine*

(Urine at pH 4·5. All determinations done in duplicate and on the same stock of urine (*P*) unless stated otherwise.)

Pb added (μg./l.)	Pb found (μg./l.)	Added Pb recovered μg./l.	Added Pb recovered %
	Method *A*		
0	208	0*	—
0†	11	0	—
180	390	182	101
360	540	332	92
500†	483	472	94
720	839	631	88
1000	1168	960*	96
1624	1776	1568	97
	Method B		
0	197	0	—
0†	0	0	—
100†	100	100	100
240	427	230*	96
333	330	330	99
480	685	488	98
1000	1043	846*	85
1400	1496	1299*	93
1800	2080	1883	105

Average recovery {Method *A* 95 %; Method *B* 97 %}

* Not done in duplicate. † Second stock of urine (*Q*).

body weight) was given as described by Carleton, Peters & Thompson (1948), and in two cases this appeared to increase the excretion of the metal as found by Ryder, Cholak & Kehoe (1947), Telfer (1947) and Ginsburg & Weatherall (1948). In case III calcium gluconate was administered in single doses of 2 g./day by injection as indicated. Sample polarograms are shown in Fig. 2, and some excretion figures in Table 3.

Urine containing low concentrations of lead

For measurements of low concentrations of urinary lead, a simple extension of method *B* would appear to lie in the use of larger samples, e.g. 125 and 25 ml. However, the precipitation of 125 ml. of urine with the amount of oxalate hitherto used results in deposition of a quantity of calcium that is often too great to dissolve in the volume of citrate buffer added to give a final volume of 2 ml. Further, the difference between the concentrations of calcium in the final solutions from the two samples of urine taken would be large. The first difficulty may necessitate the use of final volumes of 4 ml. or more, an expedient which would seriously limit the greater volumes of urine that could be treated profitably. Secondly, the disparity between the concentrations of calcium in the two final solutions introduces an error not readily assessed in any given case since the absolute quantities of calcium are not known. Thus, the use of greater volumes of urine is feasible only if the lead can be separated quantitatively in the presence of controlled amounts of calcium that do not vary greatly between the larger and the smaller urine samples.

In method *B* the first precipitation of calcium occurs in the presence of an excess of oxalate ions. It is impossible to decide whether the lead is removed from solution in these conditions by co-precipitation of sparingly soluble lead oxalate or by adsorption of

Table 2. *Variations of the diffusion current in duplicate determinations of urinary lead*

(In the first columns of figures the results of duplicate determinations and the means of these are shown. The values for Pb contents of 25 ml. and 5 ml. samples are calculated from Fig. 1, curve *B*. The results for μg. Pb/20 ml. and μg. Pb/l. urine are calculated from the means in the preceding columns.)

Method	Readings of current in galvanometer deflexion, i.e. wave height (mm.): From 25 ml. urine		From 5 mol.urine		Pb in urine sample corresponding to currents found (μg.): 25 ml. urine		5 ml. urine		Pb in urine (μg./20 ml.)	Pb in urine (μg./l.)
A	20·5	20·35	5·2	5·1	22·6	22·5	5·4	5·5	17·0	850
	20·2		5·0		22·3		5·5			
A	13·8	13·75	3·7	3·85	15·2	15·1	4·2	4·3	10·8	540
	13·7		4·0		15·0		4·4			
A	9·2	9·1	2·0	2·0	10·2	10·1	2·2	2·2	7·9	395
	9·0		2·0		10·0		2·2			
B	48·6	48·2	10·3	10·15	53·6	53·1	11·4	11·2	41·9	2095
	47·6		10·0		52·6		11·0			
B	30·2	30·1	10·5	10·4	33·4	33·3	11·6	11·5	21·8	1090
	30·0		10·3		33·2		11·4			
B	18·2	18·2	5·8	5·7	20·1	20·1	6·4	6·3	13·8	690
	18·2		5·6		20·1		6·2			
B	16·0	15·8	6·5	6·75	17·7	17·5	7·2	7·5	10·0	500
	15·6		7·0		17·2		7·7			
B	2·4	2·3	2·2	2·15	2·7	2·6	2·4	2·4	0·2	10
	2·2		2·1		2·4		2·3			

the lead ions on the calcium deposit. If the mechanism of entrainment is that of co-precipitation, the concentration of oxalate ions must be in excess of the sum of lead and calcium ion concentrations for complete removal of the lead from solution. However, if physical adsorption of lead ions on to calcium oxalate will effect the same quantitative separation, then complete removal of lead from a solution containing excess of calcium over oxalate ions should be possible. That this is so was shown in the following trial, where recoveries of lead from urine, in which

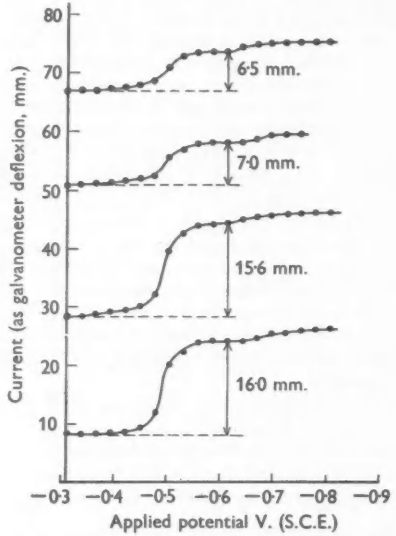

Fig. 2. Polarograms of lead from duplicate samples of pathological urine (method *B*). Upper curves 5 ml. urine. Lower curves 25 ml. urine. Applied potential measured against Standard Calomel Electrode; 10·9 mm. deflexion = 0·1 μa. Deduced value for lead content of urine is 410 μg./l.

the lead content was raised to 500 μg./l., were compared by two methods: in the first (*X*), method *B* was applied, whereas in the second (*Y*), the precipitate was formed in the presence of excess calcium over oxalate ions. Two 25 ml. samples of urine were treated with calcium chloride solution according to the foregoing methods. To (*X*) was added 2 ml. ammonium oxalate solution in the usual way; the other sample (*Y*) was treated with 2 ml. of a diluted oxalate solution whose strength had been adjusted so that calcium equivalent to 0·3 ml. stock calcium chloride solution would be precipitated. (It was found by trial that 0·3 ml. 0·225 M-calcium chloride was equivalent to 1·85 ml. 0·035 M-ammonium oxalate (theoretical volume 1·92 ml.). Therefore, as oxalate of controlled calcium equivalence, 0·035 M-ammonium oxalate, as used for washing precipitates, was employed.) The two deposits, subjected to the procedure of method *B*, gave results indicating that recovery of lead in (*Y*) was more than 90 % of that found in the comparison experiment (*X*).

Satisfactory recoveries having been obtained with a urine rich in lead by entrainment with calcium oxalate precipitates, formed by interaction of oxalate with excess calcium ions, this modification was then applied to the analysis of a normal urine containing very little lead, estimated at less than 30 μg./l. by method *B*.

Table 3. *Urinary excretion of lead in cases of suspected lead poisoning. Effects of therapy with BAL and calcium gluconate*

(*B*, BAL (1·5 mg./kg. body wt.) administered; *G*, calcium gluconate (2 g./day) administered; —, not determined.)

Day	Case I, chronic	Case II, no Pb intoxication	Case III, acute
	Pb excretion (μg./24 hr.)		
1	75	50	6000
2	100	30	950
3	—	80	1360
4	85	30	960
5	40	65	—
6	230 *B*	115	—
7	375 *B*	40	1530 *B*
8	430 *B*	108	500 *B*
9	225 *B*	35	800 *BG*
10	290 *B*	45	320 *G*
11	180 *B*	85	—*G*
12	110	140 *B*	—*G*
13	65	125 *B*	200 *G*
14	70	40	200
15	100	65	—
16	130	45	—
17	90	40	—
18	120	—	—

Samples of the urine of 125 and 25 ml. were treated with 0·5 ml. calcium chloride solution and 2 ml. of the diluted oxalate of controlled calcium-equivalence (0·036 M). The deposits were collected after 1 hr. by centrifuging, and treated according to method *B*. The second precipitation of the calcium as phosphate gave deposits of equal volumes from the two samples, and the solutions in citrate buffer gave good polarograms from which it was concluded that the urine contained originally 25 ± 5 μg./l. of lead.

Another 24 hr. sample of urine which showed no detectable content of lead when treated by method *B* gave good duplicate results when analysed by the same modified method, as shown below:

Pb current from 125 ml. sample (mm.): 4·0, 3·8, average 3·9.
Pb current from 25 ml. sample (mm.): 2·2, 2·0, average 2·1.
Current difference equivalent to Pb in 100 ml. urine ≡ 1·8 mm. Urine contained 20 μg. Pb/l.

DISCUSSION

The choice between the two methods given above depends primarily on the nature of the urine under examination. With some samples the final undissolved materials found in the quicker method have been very small, and good results were obtained. In other cases the more exacting procedure had to be followed, since the insoluble residues were larger. Some substance, provisionally identified by the half-wave potential as iron (−0·35 V., S.C.E.) (Lingane & Kerlinger, 1941), giving a wave preceding but clearly distinct from that of lead is usually observed when method *A* is applied; it is not found in method *B* and the latter may have to be used if excessive amounts of the interfering substance are present in the urine. In all cases the shorter method is adequate for ascertaining whether the excretion of lead is abnormally high, although the rare occurrence of erratic results with phosphate precipitates found by Weber (1947) and by Reed & Gant (1942) has been confirmed. Considerations of principle, mentioned below, suggest that method *B* is more reliable in certain cases. However, when both can be employed they appear in practice to be equally accurate.

It could not be shown that any of the prescribed conditions was critical. Times of precipitation, centrifuging and draining, much greater than those prescribed, did not improve results. The times finally adopted seemed reasonable, but by no means constituted a practical minimum. The corresponding intervals used by Weber (1947) and by Reed & Gant (1942) prolong the analysis unduly, e.g. the precipitates stand 2–24 hr. and drain between 0·75 and 2 hr., whereas in the work now reported the precipitates stand for 0·5 hr. at most and drain for less than 5 min. The 15 min. period for oxidation was chosen since a minimum for consistent results was found to be 10 min., and no improvement or deterioration was found when the tubes were heated almost to dryness (40 min.).

Although it is claimed (Weber, 1947) that in 20 % citric acid equally good polarograms for lead are given in the pH range 5–7, the present author has found that in 20 % citric acid adjusted to values in the range pH 5–8 with ammonia the best polarograms were given in the narrow range pH 5·0–5·4. Therefore the value pH 5·2 was chosen for the citric buffer used.

The effect of calcium in reducing slightly the diffusion current of lead has already been noted; this effect, reported qualitatively by Reed & Gant (1942) has been measured (p. 608) and found directly proportional to the concentration of calcium.

It is not certain in what form excreted lead is present in the urine, either when BAL has been administered (Spray, Stocken & Thompson, 1947) or in its absence. In the former case, presumably, a chelated sulphur-containing complex of the metal is present. However, identical results were obtained with urine excreted after injection of BAL by application of the methods to the urine both as received and also after boiling it with bromine. The complex of lead and BAL prepared *in vitro* is insoluble in the pH range 1·6–8·2 and in more alkaline solutions will not form a precipitate with phosphates. Further, the compound is insoluble in the citrate buffer used. This gives ground for preference of method *B*, since there is a risk that the alkaline precipitation of the lead may not be quantitative. Further, the oxidation in method *B* will ensure that all the lead will be soluble in citrate and that no polarographically active thiols will reach the final solution to complicate the determination by introducing unwanted waves.

The sensitivity of the polarographic determination is such that 1 μg. of lead in the final solution (2 ml.) is equivalent to a galvanometer deflexion of approximately 1 mm. The current levels between which the diffusion currents are read are drawn through five or six points lying in a straight line. Each point, after practice, is probably observed within 0·2 mm. Therefore diffusion currents may well be read off with errors of less than 0·2 mm. Since interpretation of the results is based on 20 ml. urine, the corresponding average variation is ± 10 μg. lead/l. of urine which corresponds roughly to ± 15 μg. lead/24 hr. When 125 and 25 ml. samples of urine are used, the interpretation is based on 100 ml. urine, so that the same error in the polarographic measurement is now equivalent to ± 2 μg. lead/l., i.e. ± 3 μg. lead/24 hr. The error as a percentage of the value for lead excretion will vary inversely with the latter. The overall variation in a series of determinations carried out in duplicate is shown above to be somewhat greater. The polarographic part of the determination is therefore not the limiting factor for accuracy.

The sources of greatest error appear to lie in (1) the adjustment of the volume of the final solution and (2) variations in the final concentration of calcium. When urine contains large amounts of this metal it is practicable to add calcium chloride only to the smaller sample and thus equalize to some extent the final concentrations. The uncompensated error for reagent blank thus introduced will be negligible, since the calcium chloride can be freed from lead very easily with a little oxalate before use.

A few normal urines examined showed values for lead excretion of less than 60 μg. in 24 hr. With the above methods (*A* and *B*) the determinations on urine containing less than 50 μg. lead/l. were not satisfactory, since variations between duplicates were of the same order of magnitude as the difference

in diffusion currents corresponding to the lead in 20 ml. urine. Concentrations of lead smaller than 50 μg./l. can, however, be determined by the modified procedure reported at the end of the experimental section.

No attempt has been made to use the present methods for material other than urine.

SUMMARY

1. Two relatively rapid methods for the polarographic determination of pathologically increased lead in urine are described, together with a modification for the estimation of lower concentrations.

2. The quantitative recovery obtained in the entrainment procedures of Ross & Lucas (1935) is confirmed.

3. Applications to cases of suspected lead intoxication are described; there is evidence that 2:3-dimercaptopropanol (BAL) increases the excretion of lead in the urine.

4. Estimates have been obtained for the diffusion coefficients of the complex lead ion in citrate buffer in presence and absence of calcium.

Thanks are due to Prof. R. H. S. Thompson, in whose department this investigation was carried out, for encouragement and helpful criticism and for his part in the clinical aspects of this work; also to the clinical and nursing staff and to the patients for their co-operation, and to Miss B. R. Kirk for technical assistance.

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Properties of Preparations from the Central Nervous System which Degrade Coenzymes I and II; their Connexion with Carbohydrate Metabolism

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Extraction of the system which degrades cozymase in nervous tissues, and examination of the action of various agents upon it, were considered to be of value as the system is a very potent one which acts also on coenzyme II though not on the dihydrocoenzymes (McIlwain, 1949*a*, *b*). It could thus affect the main energy-yielding reactions of the brain at several points.

The system has previously been examined in brain slices and in ground brain (for references see McIlwain & Rodnight, 1949*a*, *b*). In such preparations a large part of the activity remains firmly attached to the solid particles. There is also evidence that material with cozymase-degrading activity passes into solution in homogenates of brain in water (Utter, Wood & Reiner, 1945; Meyerhof & Wilson, 1947; McIlwain & Rodnight, 1949*a*). We have found that the active material is also soluble in strong salt solutions, and that this property, combined with its solubility in water and relative insolubility in isotonic saline, can be used to separate it from other materials.

Following the separation of the cozymase-degrading system, an examination has been made

of the action on it of various substances which affect either carbohydrate metabolism or the functional activity of nervous tissues. These studies have suggested that the system degrading cozymase is involved in the control of aerobic glycolysis.

EXPERIMENTAL

Cozymase specimens, salines, tissue preparations, and methods of determining nicotinamide and acid formation during cozymase breakdown were those previously described (McIlwain & Rodnight, 1949*a*, *b*).

Examination of cozymase-degrading activity during fractionation of the enzyme was made anaerobically at pH 7 in bicarbonate-NaCl as described by McIlwain & Rodnight (1949*a*), CO_2 evolution and nicotinamide formation being measured. From the manometric readings, the initial rate of cozymase degradation was determined graphically. The amount of material taken for estimation was so arranged that the rates measured were between 0·8 and 3 μmol./vessel/hr. Total N was determined by a micro-Kjeldahl method.

The action of various substances on cozymase degradation was assessed by adding them, together with the degrading enzyme in bicarbonate saline, to the main compartments of Warburg vessels. The side arms contained cozymase, the centre well yellow phosphorus, and the gas space 5 % (v/v) CO_2-N_2 except when otherwise described. The initial concentration of cozymase in the reaction mixture was between 4 and 6×10^{-4} M. The rate of reaction in most cases was followed by evolution of CO_2 and checked by determination of nicotinamide at the end of reaction. When the course of the reaction could not be followed by CO_2 evolution (some reagents retained CO_2, and when CO was studied, the reaction was carried out in phosphate-saline), more than one determination of nicotinamide was made in order that the rate of breakdown could be obtained.

Of the heterocyclic compounds whose actions were studied, pyridine-3-sulphonamide was prepared according to Machek (1938); dihydrocozymase (McIlwain & Rodnight, 1949*b*) according to Schlenk (1941); phenazine methosulphate, as described by Dickens & McIlwain (1938); 2-aminophenazine methosulphate, according to Kehrmann (1914); pyocyanine, according to McIlwain (1937). Nicotinic acid hydrazide was given by Dr F. L. Rose, Imperial Chemical (Pharmaceuticals) Ltd.; 3-acetylpyridine by Dr M. Gore (prepared according to Strong & McElvain, 1933); 4(5)-3′-pyridylglyoxaline by Dr G. C. Leitch (Clemo, Holmes & Leitch, 1938); the nicotinamide glycosides by Prof. A. R. Todd and Dr J. L. Haynes; ethyl red by Dr F. M. Hamer, Kodak, Ltd.; and lysergic acid diethylamide by Sandoz Products, Ltd.

RESULTS

Extraction and purification

Extraction with water. This is shown for sheep brain and ox spinal cord in Table 1.

In preparation A, sheep brain (80 g.) was rubbed for 5 min. in a mortar with an equal weight of sand, and suspended in 400 ml. 0·9 % NaCl. This was centrifuged and the residue resuspended in saline by mechanically stirring for 3 min., and again centrifuged. Three such washings were given in all, and the residue made up to 760 ml. with the saline.

Table 1. *Extraction of cozymase-degrading enzyme with water*

Starting material	Preparation examined	Activity in degrading cozymase: μmol./hr./specimen derived from 1 g. fresh weight	Activity in degrading cozymase: μmol./hr. mg. N
A. Sheep brain	Ground	145	8·5
	1st water extract	21	14·5
	2nd water extract	60	17·0
	3rd water extract	12	14·9
	Residue from extraction	3	7·6
B. Ox spinal cord	Ground and washed with 0·9 % NaCl	57	11·6
	1st water extract	1·6	7·4
	2nd water extract	4·9	4·5
	3rd water extract	21	24·4
	Residue	19·2	8·3

For extraction, 150 ml. of this suspension were centrifuged, the solid stirred with 150 ml. of water, and again collected by centrifuging. This solution, still containing saline from the original preparation, was relatively clear and had little activity. Continued washing now yielded more active, opalescent extracts, and in the preparation A of Table 1, about half the activity of the original preparation is seen to be removed by two washings. Most of the activity not accounted for (about one-third of the whole) was in the first saline washings which were discarded, and of which the activity/unit volume was small. Other preparations of brain (sheep and guinea pig), in which the volumes of washing and extracting fluids and the times of extraction were varied, gave results which differed in detail from those of Table 1, but in which the proportion and activity of the extracted material were comparable. Active material was extracted also from ox cord by this method, but somewhat less readily than from brain (Table 1).

Properties of the aqueous extracts. These were opalescent solutions containing about one-third of the nitrogen of the original preparation. Their preparation was therefore not an important step in purification. They contained no structures which could be recognized microscopically at a magnification of 1000, in distinction to the brain ground with sand in which much tissue debris, nuclei and granules were visible. The extracts retained activity with little loss during 1 month in a refrigerator. During this time, material containing about one-third of both the nitrogen and the activity precipitated spontaneously. The bulk of the remaining nitrogen and activity could be precipitated by mild acidification. Attempts to base separations on this property were not successful; acetate buffers of pH between 4 and 6 precipitated varying amounts of the solid and activity without significant enrichment.

Precipitation from aqueous solution by ethanol resulted in partial loss of activity; with acetone

neither loss nor enrichment was found. After keeping, some precipitation occurred with phosphate (0·02 M, pH 7), with a little enrichment (29 μmol./hr./mg.N; starting material, 21 μmol./hr./mg.N), but including a variable part of the initial activity. Active material was easily precipitated by sodium sulphate (80 % precipitated at 0·75 M, pH 7·5; 73 % precipitated at 0·27 M with 0·02 M phosphate, pH 7; activity, 25 μmol./hr./mg.N), but again a useful separation was not obtained. Preparation *WS* of McIlwain & Rodnight (1949*b*) was obtained in this method.

Extraction with 1–1·5 M-*sodium chloride.* In contrast to the extraction with water, this process yielded fractions of markedly increased activity. It was carried out because the extraction with water, and the properties of the water extract, suggested that the activity of the preparation was associated with nucleoprotein-like materials. Whether this is correct or not, it was found that after nervous tissue had been washed with isotonic saline, with the removal of some cozymase-splitting activity, extracts containing more of such activity could be obtained by washing with 1·2 M-NaCl (Table 2). A proportionately smaller quantity of nitrogenous material was extracted, and the activity of the preparations in μmol. cozymase split/hr./mg. N therefore rose. In this respect, spinal cord was a more satisfactory starting material than brain; from cord, preparations with activities of 100–150 μmol. cozymase split/hr./mg. N were obtained.

Table 2. *Extraction of cozymase-degrading system with* 1·2 M-*sodium chloride*

Starting material	Preparation examined	Activity in degrading cozymase: μmol./hr./specimen derived from 1 g. fresh weight	Activity in degrading cozymase: μmol./hr./mg. N
A. Guinea pig brain	Ground	210	13·0
	3 washings with 0·9 % NaCl	25	3·3
	1st 1·2 M-NaCl extract	44	25
	2nd 1·2 M-NaCl extract	29	30
	Residue	72	16·5
B. Ox spinal cord	Ground and washed with 0·9 % NaCl	102	12·0
	0·9 % NaCl washings	47	21·3
	1st 1·2 M-NaCl extract	14	100
	2nd 1·2 M-NaCl extract	8	106
	Residue	22	4·3
C. Ox spinal cord	Blended	120	10·4
	1st 1·2 M-NaCl extract	22	72
	2nd 1·2 M-NaCl extract	20	129
	3rd 1·2 M-NaCl extract	6	32
	Residue	20	5·4

For preparation B, Table 2, ox cord, brought from a slaughter-house in ice, was passed through a cold meat mincer, rubbed with sand as above, and passed through muslin to remove membranes. It was suspended in 1 l. 0·9 % NaCl, stirred for 5 min., centrifuged, and the residue resuspended and washed twice as before. The residue was then extracted by stirring with 1·2 M-NaCl (1 l.) for 30 min. at 10°, centrifuged, and the residue treated again in the same way. For preparation *C*, 200 g. cord were blended (Waring) for 12 sec. with 200 ml. 0·9 % NaCl, a further 800 ml. added, and the mixture stirred for 15 min. After centrifuging, the washing was twice repeated; extraction with 1·2 M-NaCl was done as above.

Properties of sodium chloride extracts. In order to test for cozymase degradation, most of the sodium chloride was removed by dialysis in cellophan bags against rapidly running tap water, at 10° for 2 hr. During this time, inactive material precipitated. Relatively little nitrogenous material passed out of the bags; this is understandable, as prior to extraction the tissue had been well washed with 0·9 % sodium chloride. No morphological structure was visible in the final preparations. These constituted the preparations *SsDC* referred to by McIlwain & Rodnight (1949*b*).

Some enzymic properties of preparations degrading cozymase

Many enzymes of brain tissue are lacking in the washed ground brain or cord used as the source of the degrading enzyme, for the reaction with cozymase could be followed satisfactorily by manometric methods in the presence of lactate, pyruvate, glucose, glutamate, malate, or ethanol. These compounds therefore underwent no changes leading to changes in acid or gas under the conditions of determining cozymase breakdown.

Enzymes hydrolysing adenosine triphosphate are, however, known to be associated with particulate matter in brain homogenates (Meyerhof & Wilson, 1947). Because of their relevance to carbohydrate metabolism, we have determined the rate of breakdown of adenosine triphosphate by preparations degrading cozymase (Table 7). Washed brain preparations catalysed a rapid breakdown of adenosine triphosphate with formation of inorganic phosphate, loss of acid-labile phosphate, and formation of acidic groupings which liberated some carbon dioxide from bicarbonate. Their ability to do this was, however, almost entirely lost during the extraction with strong salt and the dialysis, by which the cozymase degrading system was concentrated (Table 7).

Effects of substances on cozymase degradation

General. The actions of several substances have been assessed quantitatively with different preparations of the degrading enzyme. The action of nicotinamide on cord preparations which were simply washed with 0·9 % sodium chloride was compared with its action on an extracted and dialysed enzyme from the same source. No marked difference was found (Table 3). The action of phenosafranine on the activity of a cord preparation was similar to that of its action on the activity of an extract from

brain (Table 5). The actions of potassium and calcium chlorides, the pyridylglyoxaline, neutral red, and phenosafranine were also examined on a variety of preparations which included specimens from guinea pig, rabbit, and sheep brains, and no major differences were found which were specific to the preparations.

Table 3. *Inorganic compounds and pyridine derivatives on cozymase degradation*

Substance	Concentration in reaction mixture (M)	Enzyme preparation*	Rate of reaction (% of rate without added substance)
KCl	10^{-1}	*C, SsDC*	96
$CaCl_2$	2×10^{-2}	*C, SsDC*	100
NaF	33×10^{-3}	*B, W*	105
KCN	33×10^{-3}	*B, W*	100
$K_3Fe(CN)_6$	$1{\cdot}67 \times 10^{-3}$	*B, W*	106
$K_4Fe(CN)_6$	$1{\cdot}67 \times 10^{-3}$	*B, W*	96
KI	$3{\cdot}3, 0{\cdot}67 \times 10^{-3}$	*B, W*	107, 102
Nicotinamide	$2{\cdot}67 \times 10^{-3}$	*B, W*	27
Nicotinamide	$1{\cdot}33 \times 10^{-3}$	*B, W*	44
Nicotinamide	$6{\cdot}67 \times 10^{-4}$	*B, W*	60
Nicotinamide	$3{\cdot}33 \times 10^{-4}$	*B, W*	90
Nicotinamide	$1{\cdot}33 \times 10^{-3}$	*C, E*	42
Nicotinamide	$6{\cdot}67 \times 10^{-4}$	*C, E*	54
Nicotinamide	$3{\cdot}33 \times 10^{-4}$	*C, E*	78
Nicotinic acid hydrazide	$3{\cdot}3 \times 10^{-3}$	*C, SsDC*	108
Coramine	$3{\cdot}3 \times 10^{-3}$	*B, W*	95
3-Acetylpyridine	$1{\cdot}1 \times 10^{-2}$	*C, SsDC*	35
3-Acetylpyridine	$1{\cdot}7 \times 10^{-3}$	*C, SsDC*	100
Pyridine-3-sulphonamide	$8{\cdot}3 \times 10^{-3}$	*B, W*	104
Nicotine	$1{\cdot}67 \times 10^{-2}$	*B, W*	24
Nicotine	$1{\cdot}67 \times 10^{-3}$	*B, W*	47
Nicotine	$1{\cdot}67 \times 10^{-4}$	*B, W*	65
4(5)-3′ Pyridylglyoxaline	$8{\cdot}3 \times 10^{-3}$	*B, W*	<5
4(5)-3′ Pyridylglyoxaline	$1{\cdot}67 \times 10^{-3}$	*B, W*	5
4(5)-3′ Pyridylglyoxaline	$3{\cdot}3 \times 10^{-4}$	*B, W*	14
4(5)-3′ Pyridylglyoxaline	10^{-4}	*B, W*	53
4(5)-3′ Pyridylglyoxaline	3×10^{-5}	*B, W*	79
4(5)-3′ Pyridylglyoxaline	10^{-5}	*B, W*	95
Trigonelline chloride	$8{\cdot}3 \times 10^{-3}$	*B, W*	105
Nicotinamide methiodide	$3{\cdot}3 \times 10^{-3}$	*C, W*	97
Nicotinamide riboside	$3{\cdot}3 \times 10^{-3}$	*C, W*	85
Nicotinamide glucoside	$3{\cdot}3 \times 10^{-3}$	*C, W*	78
Dihydrocozymase	4×10^{-4}	*B, W*	98

* *B*, brain from guinea pig or sheep; *C*, ox spinal cord; *W*, washed with 0·9 % NaCl; *E*, extracted with water; *SsDC* prepared by extraction with strong salt, dialysed and centrifuged. Details of preparation are given in the previous part of this paper. Differences of less than 6 % in the rates are not considered significant.

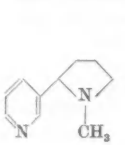
Inorganic salts. The effects of Ca^{++} and K^+ were examined because these ions affect nervous activity, and Ca^{++} had been reported by Swingle, Axelrod & Elvehjem (1942) to accelerate cozymase breakdown in homogenized rat liver, though Spaulding & Graham (1947) did not find an action in ground brain. No effect of Ca^{++} and K^+ had previously been found in this laboratory on cozymase degradation in sodium chloride washed ground sheep brain, and no action has now been found (Table 3) on the breakdown by extracted enzyme. In addition to the experiment of Table 3, the effect of adding potassium chloride (0·1 M) to a reaction mixture in a balanced physiological saline was examined, but again no change was caused. This concentration of potassium chloride causes full release of aerobic glycolysis in brain (Ashford & Dixon, 1935; Dickens & Greville, 1935).

Fluoride and cyanide ions, also found to be without action on cozymase degradation, were examined since they are frequently used as reagents. It was necessary to know the effects of iodides and of ferri- and ferro-cyanides (all found inactive) in order to control experiments in which these reagents were employed.

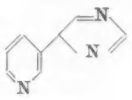
3-Substituted pyridine derivatives. Inhibition by nicotinamide is already well known (Mann & Quastel, 1941). From Table 3 it is seen to be representative of the inhibitory powers of certain 3-substituted pyridine derivatives which include nicotine, 4(5)-3′-pyridylglyoxaline (for structures, see Table 4) and 3-acetylpyridine. Of these compounds, 3-acetylpyridine was not a potent inhibitor; it would be necessary for the compound to be greatly concentrated in tissues if the present inhibition is the basis of the growth effects observed by Woolley (1945). Both nicotine and nicotinamide were more active, inhibiting the breakdown of 6×10^{-4} M-cozymase by 50 % at about 10^{-3} M. The pyridylglyoxaline was much more potent as an inhibitor, producing comparable effect to nicotinamide at about 0·1 of the concentration.

Relatively small changes in the structure of nicotinamide produced substances of greatly reduced potency. Thus, nicotinic acid, pyridine-3-sulphonamide, and nicotinic acid hydrazide and its diethylamide (coramine) showed no activity at concentrations equal to or greater than that at which nicotinamide reduced the reaction to 20 % of its uninhabited velocity. Quaternary salts of nicotinamide were also less active: nicotinamide methiodide very much so, while the riboside and glucoside had relatively small activities which were not clearly differentiated from that due to nicotinamide in the preparations. These relationships between structure and activity are to be contrasted with those of phenazine derivatives, among which quaternary salts were the more potent inhibitors. It was noted

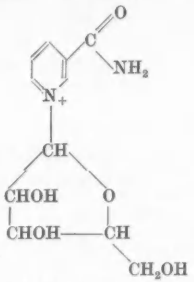
Table 4. *Some compounds whose action on cozymase degradation has been examined*



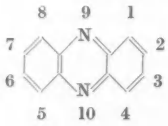
(I) Nicotine



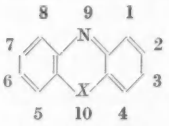
(II) 5(4)-3′ Pyridyglyoxaline



(III) Nicotinamide riboside



(IV) Phenazine



(V) Oxazine or thiazine

Substance	Basic Formula	Substituents in position 2	3	6	7, 8	10
Neutral red	(IV)	Me	NH_2	NMe_2	—	—
Neutral violet	(IV)	$NH.C_6H_4NMe_2$	NH_2	NMe_2	—	—
Phenosafranine	(IV)	—	NH_2	NH_2	—	$C_6H_5.Cl$
Janus green	(IV)	—	$N_2C_6H_4NMe_2$	NH_2	—	$C_6H_5.Cl$
Rosinduline	(IV)	—	—	NH_2	C_4H_4	$C_6H_5.Cl$
Methylene blue	(V), $X=S$	—	$NMe_2.Cl$	NMe_2	—	—
Thionine blue	(V), $X=S$	—	$NMeEt.Cl$	NMe_2	—	—
Brilliant cresyl blue	(V), $X=O$	—	$NH_2.Cl$	NEt_2	—	—

previously (McIlwain & Rodnight, 1949*b*) that dihydrocoenzymes I and II had little effect on the breakdown of cozymase, while coenzyme II competed with coenzyme I.

Alkaloids with fused pyridine or piperidine rings. Many such substances were examined without finding a potent inhibitor. The relatively small activities of quinine and strychnine are recorded in Table 6. Cinchonidine, atropine, emetine, and bulbocapnine at 3×10^{-3} M were without action on the breakdown catalysed by washed brain preparations. Lysergic acid diethylamide was similarly inactive at 3×10^{-4} M, and narcotine in a saturated solution in 0·9 % sodium chloride was also without action.

Phenazine derivatives and related compounds. Investigation of these compounds was centred round the observation (McIlwain, 1949*a*, *b*) that phenosafranine, a diamino-*N*-phenyl phenazonium salt, was a potent inhibitor of cozymase degradation. Phenosafranine had been chosen for examination on account of Dickens's (1936) observation of its release of aerobic glycolysis in brain-cortex slices and in other preparations. Related compounds were accordingly examined (Table 5; for structures see Table 4). A simple phenazonium salt, phenazine methosulphate, was much less active. Introduction of an amino group, in α-aminophenazine methosulphate, increased the activity. The keto group of pyocyanine had little effect. The two amino groups of neutral red are in the same positions as those of phenosafranine, although neutral red lacks the quaternary phenyl group (it is also methylated); neutral red is more active than the preceding compounds, but less so than phenosafranine. Neutral violet, with an additional nuclear amino group, but still lacking the quaternary phenyl group, possessed activity of the same order as that of neutral red.

Rosinduline and Janus green are both, like phenosafranine, aminophenazonium salts. Rosinduline was less active than neutral red; it however contains only one amino group, and also a fused benzene ring which does not appear in phenosafranine. Janus green is produced from phenosafranine by diazotizing and coupling it with dimethylaniline; its activity was found to be only slightly less than that of phenosafranine.

Methylene blue, thionine blue, and brilliant cresyl blue possess, instead of the phenazine, oxazine or thiazine nuclei, substituted similarly to the phena-

zines studied but lacking a quaternary phenyl group. Their activities were not large, all being less potent than the aminophenazine methosulphate.

Table 5. *Action of some phenazine, thiazine and oxazine derivatives on cozymase degradation*

Substance	Concentration in reaction mixture (M)	Enzyme preparation (see Table 3)	Rate of reaction (% of rate without added substance)
Phenazine methosulphate	$3{\cdot}3 \times 10^{-4}$	*C, W*	88
Aminophenazine methosulphate	$3{\cdot}3 \times 10^{-4}$	*C, W*	56
Pyocyanine	10^{-3}	*C, SsDC*	80
Pyocyanine	10^{-4}	*C, SsDC*	88
Neutral red	$1{\cdot}67 \times 10^{-3}$	*G, B, W*	44
Neutral red	$1{\cdot}67 \times 10^{-4}$	*G, B, W*	58
Neutral red	$3{\cdot}3 \times 10^{-5}$	*G, B, W*	78
Neutral red	10^{-4}	*C, SsDC*	65
Neutral red	10^{-5}	*C, SsDC*	81
Neutral violet	$3{\cdot}3 \times 10^{-4}$	*C, W*	61
Phenosafranine	$1{\cdot}33 \times 10^{-3}$	*B, W*	16
Phenosafranine	$3{\cdot}3 \times 10^{-4}$	*B, W*	13
Phenosafranine	$6{\cdot}7 \times 10^{-5}$	*B, W*	38
Phenosafranine	$1{\cdot}3 \times 10^{-5}$	*B, W*	88
Phenosafranine	10^{-4}	*C, SsDC*	28
Phenosafranine	10^{-5}	*C, SsDC*	85
Janus green	10^{-4}	*C, SsDC* (*a*)	31
Janus green	10^{-5}	*C, SsDC* (*a*)	78
Janus green	3×10^{-4}	*C, SsDC* (*b*)	28
Janus green	10^{-4}	*C, SsDC* (*b*)	38
Janus green	3×10^{-5}	*C, SsDC* (*b*)	75
Janus green	10^{-5}	*C, SsDC* (*b*)	95
Rosinduline	$3{\cdot}3 \times 10^{-4}$	*C, W*	78
Methylene blue	$3{\cdot}3 \times 10^{-4}$	*C, W*	98
Thionine blue	5×10^{-4}	*C, SsDC*	70
Thionine blue	$1{\cdot}67 \times 10^{-4}$	*C, SsDC*	95
Thionine blue	5×10^{-5}	*C, SsDC*	103
Brilliant cresyl blue	10^{-3}	*C, SsDC*	55
Brilliant cresyl blue	10^{-4}	*C, SsDC*	100

It thus appeared that while some activity in inhibiting cozymase degradation was possessed by a variety of phenazine and structurally similar substances, activity was greatest in the phenazines. This activity was increased by basic substituents, and by forming quaternary salts. Sufficient analogues were not examined for the significance of the individual groups in phenosafranine to be assessed.

Other substances affecting aerobic phosphorylation or aerobic glycolysis in brain-cortex slices. Phenosafranine and nicotine are especially interesting in affecting both phosphorylation and glycolysis, and accordingly the behaviour towards cozymase degradation of other substances which affect these processes has also been examined (Table 6). Carbon monoxide, like cyanide, was without effect on the breakdown. The experiment with carbon monoxide used an atmosphere of 100 % carbon monoxide, and light was excluded from the experimental vessel. The guanidines and diamidines tested were also inactive at concentrations much higher than those which affect glycolysis (Dickens, 1939). Ethyl red was inhibitory only in relatively high concentrations, and styryl 430 and benzylviologen little if at all in the range examined (cf. Dickens, 1936).

Table 6. *Action on cozymase degradation of some substances affecting aerobic glycolysis; miscellaneous compounds*

Substance	Concentration in reaction mixture (M)	Enzyme preparation (see Table 3)	Rate of reaction (% of rate without added substance)
Carbon monoxide	(in equilibrium with 1 atm. in gas phase)	*C, SsDC*	93
Guanidine	10^{-2}, 10^{-3}	*C, SsDC*	100, 104
Methylguanidine	10^{-2}	*C, SsDC*	70
Methylguanidine	10^{-3}	*C, SsDC*	100
Synthalin B	10^{-3}	*C, SsDC*	97
Synthalin B	10^{-4}	*C, SsDC*	102
Undecane diamidine	10^{-3}	*C, SsDC*	102
Undecane diamidine	10^{-4}	*C, SsDC*	95
Ethyl red	$3{\cdot}3 \times 10^{-4}$	*C, W*	38
Ethyl red	$6{\cdot}7 \times 10^{-5}$	*C, W*	104
Ethyl red	5×10^{-5}	*C, SsDC*	90
Ethyl red	10^{-5}, 5×10^{-6}	*C, SdDC*	96
Styryl 430	10^{-4}, 10^{-5}	*C, SsDC*	94, 99
Benzylviologen	10^{-4}, 10^{-5}	*C, SsDC*	97
2:4-Dinitrophenol	$1{\cdot}66 \times 10^{-3}$	*C, SsDC*	55
2:4-Dinitrophenol	$1{\cdot}66 \times 10^{-4}$	*C, SsDC*	65
Quinine	$3{\cdot}3 \times 10^{-3}$	*B, W*	89, 95
Strychnine	$3{\cdot}3 \times 10^{-3}$	*B, W*	75, 90
Benzimidazole methiodide	$3{\cdot}3 \times 10^{-3}$	*B, W*	99

Miscellaneous materials. Many naturally occurring materials were without action on the degradation catalysed by washed brain or cord: ethanol (3×10^{-3} M), lactate (3×10^{-3} M), pyruvate (5 and 20×10^{-3} M), malate (3×10^{-3} M), histamine (2×10^{-2} M), mescaline (8×10^{-3} M), eserine ($3{\cdot}3 \times 10^{-3}$), adenosinetriphosphate (5×10^{-3} M), insulin (13 units/ml.) and protamine (0·3 and 1 mg./ml.). Three specimens of normal human serum (1 ml./3 ml. reaction mixture) were without action on the breakdown by a washed-cord preparation, as also were two extracts from sheep brain (1 ml./3 ml.). These were prepared (*a*) by blending the brain with an equal volume of water, heating to 100° for 15 min., and centrifuging; (*b*) with sodium sulphate according to the method of Deutsch, Eggleton & Eggleton (1938). Metrazol ($3{\cdot}3 \times 10^{-3}$ M) was also inactive.

Lack of action of phenosafranine on breakdown of adenosinetriphosphate by brain homogenates

Washed particles from brain homogenates react also with adenosinetriphosphate (p. 614). Phenosafranine has been found, however, to have no action on this process. Table 7 shows that any effect of 3×10^{-5} M-phenosafranine is within the limits of

Table 7. *Comparison of breakdown of adenosinetriphosphate and cozymase by preparations from nervous tissue; lack of action of phenosafranine on the reaction with adenosinetriphosphate*

(Reactions were carried out anaerobically as described for cozymase degradation in the experimental section, except that the reaction mixture was in balanced bicarbonate saline (Krebs & Henseleit, 1932). Labile phosphate was determined as the difference in inorganic phosphate (Lowry & Lopez, 1946), before and after heating for 7 min. in N-HCl.)

Preparation (see Table 3)	Additions (μmol./3 ml.)	Change in labile phosphate (μmol./ml.)	Period (min.)	CO_2 evolved from bicarbonate (μmol./vessel)	Period (min.)	Initial rate (μmol/vessel/hr.)
B, W	Adenosinetriphosphate, 18	1·39	60	1·34	60	3·76
B, W	Adenosinetriphosphate, 18 phenosafranine, 0·1	1·35	60	1·26	60	3·70
B, W	Cozymase, 1·5	—	—	0·65	80	0·8
C, SsDC	Adenosinetriphosphate, 18	0·2	120	0·08	120	0·05
C, SsDC	Cozymase, 1·5	—	—	0·5	120	1·0

measurement, whereas this concentration inhibits cozymase degradation in the same preparations by about 50 %. Phenosafranine at an initial concentration of 3×10^{-4} M was also without effect on the breakdown of adenosinetriphosphate.

DISCUSSION

The outstanding finding of the present survey is of the potent action of phenosafranine on cozymase degradation. The effect of this substance was examined because its action in disturbing the normal course of carbohydrate breakdown in brain cortex had remained without explanation since being discovered by Dickens (1936). The disturbance caused by phenosafranine consists of an increase in aerobic glycolysis to values approaching anaerobic levels, without much effect on respiration. It thus constitutes a disturbance of the Pasteur effect. It is remarkable that Dickens suggested in 1936 that the action of compounds like phenosafranine might be through processes in which coenzymes, including cozymase, took part. Phenosafranine has now been found to be the most potent of a group of phenazine compounds in its action on the enzyme system degrading cozymase, but this property is shared by other compounds. When comparison was made (McIlwain, 1949*a*) between the actions of these compounds on the degradation of cozymase, and on release of aerobic glycolysis, some correlation appeared to exist. The correlation was, however, complicated by concentration gradients of the substances between solution and tissue and is being made the subject of separate studies.

At this point it was clear that valuable evidence on the possible connexion between aerobic glycolysis and cozymase degradation would be given, if entirely different types of substances affected both processes. It has now been found that such evidence already exists in observations of previous workers, though these have not before been interpreted in this fashion; and the evidence has also been extended by the observations of the present paper.

Baker, Fazekas & Himwich (1938), in determining whether glucose oxidation in brain passed through lactic acid, searched for differential inhibitors of metabolism of the two substrates and found nicotine, nicotinic acid and nicotinamide to inhibit differentially. In addition, however, nicotinamide at 0·03 M is shown from Table 3 of that paper to have only a small action on the respiratory rate of slices of rat cerebral cortex, but consistently to increase their aerobic lactic acid formation. The authors, in the text, minimized this observation as other compounds were more potent in showing both this effect and the differential action in which they were interested. However, as stated previously, nicotinamide was later shown to inhibit cozymase degradation and the compound thus afforded a connexion between cozymase metabolism and aerobic glycolysis of the same type as that afforded by phenosafranine.

Moreover, it has been observed that the same correlation extended to nicotine. Baker *et al.* (1938) found considerable increase in aerobic glycolysis of cat cerebral cortex by 0·03 M-nicotine, and an inhibition of respiration of 28 %. With guinea pig-brain cortex, we find an increase in glycolysis with lesser inhibition of respiration. As shown above, nicotine inhibits cozymase degradation. The correlation also extended to 4(5)-3′-pyridylglyoxaline (McIlwain, 1949*b*) a substance which is structurally similar to nicotine, but whose action on either cozymase degradation or aerobic glycolysis had not previously been studied. The pyridylglyoxaline was found to be more potent than nicotine in its action on both these processes.

Reviewing the results on inhibition of cozymase degradation, it is seen that all effective inhibitors of the breakdown are reported to increase aerobic glycolysis. Many compounds affect neither process. The structural criteria for effective inhibition of

cozymase degradation were fairly exacting, as all the potent inhibitors of the reaction belonged to the groups of phenazine and pyridine derivatives, though many others were examined.

Although all substances inhibiting cozymase degradation released aerobic glycolysis, many substances reported to release aerobic glycolysis were without effect on cozymase degradation: potassium chloride, carbon monoxide and the guanidines and amidines were outstanding in this respect. This is a finding to be expected when the action of substances on an organized series of reactions involving a dozen or more enzymes is compared with their actions on a single process of the series. Whether the reactions of carbohydrate degradation and utilization, as they operate in tissues, involve the continual breakdown and resynthesis of cozymase is unknown; but the above evidence strongly indicates that the enzyme isolated from the tissue which degrades cozymase does play a specific role in carbohydrate metabolism in brain. The mechanism of this participation appears likely to be through an effect on the phosphorylation normally accompanying oxidation, for Judah & Williams-Ashman (1949) found that phenosafranine inhibits the phosphorylation accompanying pyruvate oxidation in rat-liver homogenates, and phenosafranine has now been found in these laboratories (Case & McIlwain, unpublished) to have a similar effect with brain.

SUMMARY

1. The system degrading cozymase in preparations of mammalian brain and spinal cord has been extracted by water or by strong salt solutions, and separated from some 90 % of the nitrogenous constituents of the tissues.

2. Of about sixty-five materials which were examined for their actions on the breakdown, none caused significant stimulation even with a washed and dialysed enzyme preparation.

3. Two main groups of compounds inhibited the breakdown when present in concentrations comparable to, or less than, those of the cozymase undergoing reaction. These were pyridine and phenazine derivatives.

4. The active pyridine compounds included nicotine and 4(5)-3′-pyridylglyoxaline as well as nicotinamide. Of the phenazine derivatives, phenosafranine and Janus green were most potent.

5. Correlations existed between the actions of these substances on cozymase degradation and their reported actions on the release of aerobic glycolysis by brain slices.

I am greatly indebted to Mr R. Rodnight and Mr I. Grinyer for assistance during part of these experiments, and to those named above who gave specimens of some of the compounds studied.

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Concentration or Exclusion of Some Heterocyclic Compounds by Brain Cortex, in Relation to their Effects on Carbohydrate Metabolism

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Some pyridine and phenazine derivatives which disturb the normal balance of carbohydrate metabolism in brain-cortex slices inhibit the degradation of cozymase (McIlwain, 1949). To assess the relation between the two actions of such compounds, it was decided to determine the concentrations at which a number of them acted on each process. The two processes have however been studied under different conditions. Degradation of cozymase has been followed in homogenates and extracts of tissues from the central nervous system, and the actions of the various compounds upon it are described elsewhere (McIlwain, 1950). The reaction is slower and of less constant rate in brain slices, which cozymase does not appear to enter (Gore, Ibbott & McIlwain, 1950).

The balance of carbohydrate metabolism, on the other hand, has been studied in brain slices. Physiological experiments with resting men or dogs, breathing air, show that *in vivo* the brain converts to lactic acid about 10–15 % of the glucose which it utilizes; almost all the remainder is oxidized completely (Gibbs, Lennox, Nims & Gibbs, 1942; Himwich & Himwich, 1946). The proportion converted to lactic acid increases in hypo-oxia and in certain other conditions. A similar balance between glycolysis and oxidation is maintained in brain slices, and, in particular, their glycolysis is high anaerobically but suppressed in air; they thus exhibit the Pasteur effect (see, for example, the review of Burk, 1939). In homogenates, however, this is not necessarily the case (Reiner, 1947).

During this study it became evident that in comparing the action of substances in homogenates and in slices, the interaction of slice and added substance must be examined in detail. One of the added substances was found to reach concentrations in the slices a hundred times those in the surrounding medium, while in another case, the ratio was 0·2. To assist in the interpretation of such findings, factors conditioning the entry of substances to the slices were studied. The concentrations of the added substances in the slices were then determined during experiments in which their effects on respiration and aerobic glycolysis were being studied.

METHODS

Tissue slices. Guinea pigs were killed by a blow on the neck, sometimes bled, and the brain removed and sliced, while moistened with the saline used in the latter part of the experiment. Slices of cortex were cut with a recessed microscope slide as guide and washed into a dish of the appropriate saline. When sufficient slices for an experiment had been cut, they were drained free from saline, weighed on a torsion balance, and dropped into Warburg vessels which already contained all other reagents. Slices weighing 50–60 mg. were commonly used and the outer ones were rejected. The dry weight was found to be 0·13 of the moist weight of slices handled in this way; weights of tissue quoted in the text are moist weights.

Salines. Phosphate saline contained 134 mM-NaCl; 5·4 mM-KCl; 1·34 mM-KH_2PO_4, $MgSO_4$, and $CaCl_2$; 13 mM-glucose; 10·4 mM-Na_2HPO_4 brought to pH 7·4 by HCl; it was saturated with O_2. Bicarbonate saline contained 124 mM-NaCl; 5 mM-KCl, 1·24 mM-KH_2PO_4, and $CaCl_2$; 12 mM-glucose and 26·1 mM-$NaHCO_3$, and was equilibrated with 5 % CO_2 in O_2.

Respiration and aerobic glycolysis. Respiration was determined with Warburg monometers and vessels with a centre well containing KOH paper. The total lactic acid formation was obtained at the end of experiments by Barker & Summerson's (1941) method, and the course of its formation was determined manometrically by calculation from the respiration and the net gas exchange in vessels with bicarbonate saline and 5 % CO_2-O_2. The choice of this method and its applicability to the present experiments are assessed below.

Determination of the heterocyclic compounds studied. Several of these were coloured and were determined in a photoelectric absorptiometer, both in the solution and in the tissue after metabolism. The solution was centrifuged if necessary to free it from tissue debris and compared with a solution of the compound concerned, in buffer of the same pH as that from the metabolic mixture. The slices were drained from liquid and rubbed in a tube and glass rod, with five times their weight of anhydrous Na_2SO_4. The added compound was then extracted from the dried mixture with 2–5 portions of 0·6–1·5 ml. of $CHCl_3$, according to the nature and amount of pigment. The total extract was weighed, or made up to a standard volume, and compared photoelectrically with standard solutions of the pigment in $CHCl_3$. Slices without pigment were extracted similarly as controls. In the photoelectric comparisons, Ilford spectrum filter

no. 603 was used for α-aminophenazine methosulphate and neutral red; no. 604 for phenosafranine; no. 605 for ethyl red; no. 607 for diazine green and pyocyanine; and no. 608 for brilliant cresyl blue and thionine blue.

Nicotine was determined by Trim's (1948) method. Estimation of nicotinamide followed previous practice (McIlwain & Rodnight, 1949); it was extracted from slices by grinding in the $ZnSO_4$ used for deproteinizing, after which NaOH was added to neutrality, the whole diluted to a known volume, centrifuged, and samples of the supernatant taken for estimation. The pyridine derivatives, in the concentrations in which they were studied, were found to be partly excluded from slices. In determining their concentration in slices, it was especially necessary to examine the conditions chosen for the separation of slice and solution. Results obtained by simple draining and by one or by two washes of 1 and 3 sec. in saline were therefore compared. Differences between them were small.

RESULTS

Entry of heterocyclic compounds into slices

Course of entry aerobically

This was first examined under normal metabolic conditions: aerobically, with glucose, in a balanced physiological saline. The entry was found to be gradual, and to take between 30 and 80 min. to reach an approximately stable value. Such observations are illustrated in Fig. 1 with respect to phenosafranine, neutral red, diazine green, and ethyl red at concentrations about those at which they exert their metabolic effects. The quantity in the tissue was found in each case to be stable between 60 and 120 or 150 min.

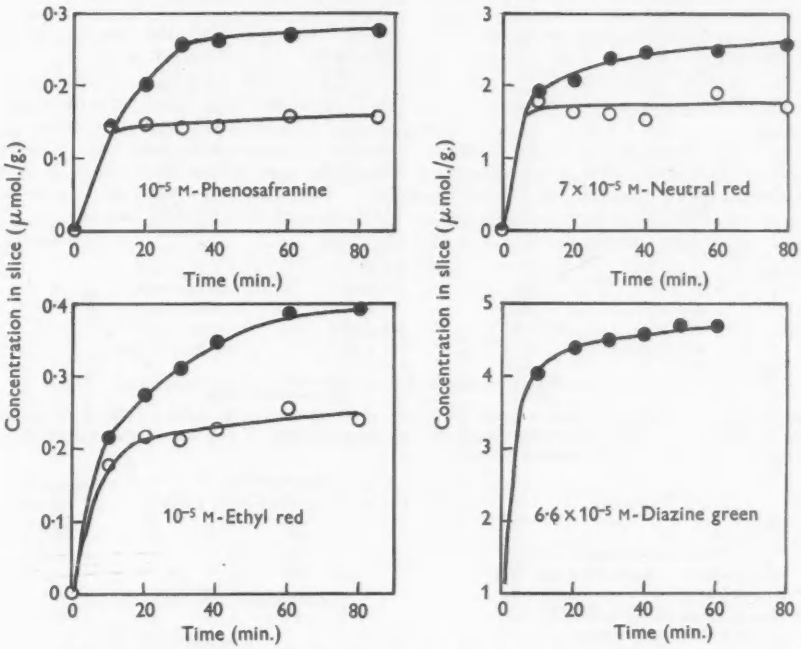

Fig. 1. Course of absorption of some heterocyclic compounds by guinea pig-brain cortex. Aerobically, with glucose: ●—●. Anaerobically, no glucose: ○—○. Slice wt./ml. of saline: 15–25 mg. Concentrations quoted are those in solution at the beginning of the experiment.

The experiments of Fig. 1 showed that when equilibrium was reached, some 60–90 % of the added substance had left the medium for the tissue slice. As the slice was only about one-fiftieth of the volume of the medium, this indicated a very considerable concentration of the substance in the tissue. The manner in which the added substances were distributed between medium and tissue was therefore assessed quantitatively at different concentrations; these results are given in Table 1. From this it is seen that the concentration of most of the added substances in the tissue reached 50 to 300 times that of the substance remaining in solution. The ratios of the two concentrations in some cases tend to vary with change in the initial amount of added substance, and in others to be independent of this.

Table 1. *Concentration of substances in saline and tissue when equilibrium is reached or approached*

(Experiments were aerobic, lasting 80–100 min., and with about 20 mg. tissue/ml. solution. Diazine green not attached to tissue was found to be partly precipitated. The values quoted for the solution were obtained after removing such precipitated material.)

Initial solution			Final concentrations of added compounds (M)		Ratio of concentrations: tissue/solution
Added compound (M)		Saline (*B*, bicarbonate; *P*, phosphate)	Solution	Tissue	
Phenosafranine	10^{-5}	*B*	$0{\cdot}38 \times 10^{-5}$	$3{\cdot}16 \times 10^{-4}$	83
	$1{\cdot}08 \times 10^{-5}$	*P*	$0{\cdot}49 \times 10^{-5}$	$3{\cdot}4 \times 10^{-4}$	70
	5×10^{-5}	*B*	$1{\cdot}7 \times 10^{-5}$	$9{\cdot}55 \times 10^{-4}$	56
	5×10^{-5}	*P*	$1{\cdot}6 \times 10^{-5}$	$9{\cdot}65 \times 10^{-4}$	60
Diazine green	$2{\cdot}5 \times 10^{-5}$	*P*	$0{\cdot}95 \times 10^{-5}$	$3{\cdot}7 \times 10^{-4}$	40
	$2{\cdot}5 \times 10^{-5}$	*B*	$0{\cdot}90 \times 10^{-5}$	$4{\cdot}3 \times 10^{-4}$	48
	$7{\cdot}5 \times 10^{-5}$	*P*	$1{\cdot}1 \times 10^{-5}$	12×10^{-4}	109
	$7{\cdot}5 \times 10^{-5}$	*B*	$1{\cdot}05 \times 10^{-5}$	15×10^{-4}	145
Neutral red	5×10^{-5}	*B*	$0{\cdot}5 \times 10^{-5}$	$7{\cdot}65 \times 10^{-4}$	153
	10^{-4}	*B*	$1{\cdot}2 \times 10^{-5}$	$1{\cdot}53 \times 10^{-3}$	130
	3×10^{-4}	*B*	$2{\cdot}55 \times 10^{-5}$	$3{\cdot}13 \times 10^{-3}$	120
Ethyl red	10^{-6}	*B*	$<10^{-7}$	$7{\cdot}5 \times 10^{-5}$	>750
	$3{\cdot}3 \times 10^{-6}$	*B*	10^{-6}	$1{\cdot}5 \times 10^{-4}$	150
	$3{\cdot}3 \times 10^{-6}$	*P*	10^{-6}	$1{\cdot}38 \times 10^{-4}$	138
	10^{-5}	*B*	$1{\cdot}12 \times 10^{-6}$	$3{\cdot}4 \times 10^{-4}$	300
Nicotinamide	2×10^{-2}	*P*	2×10^{-2}	$1{\cdot}37 \times 10^{-2}$	0·69
	2×10^{-2}	*B*	2×10^{-2}	$1{\cdot}40 \times 10^{-2}$	0·70
	5×10^{-2}	*P*	5×10^{-2}	$3{\cdot}80 \times 10^{-2}$	0·76
Nicotine	$3{\cdot}3 \times 10^{-2}$	*P*	$3{\cdot}3 \times 10^{-2}$	$0{\cdot}73 \times 10^{-2}$	0·22
	$8{\cdot}3 \times 10^{-2}$	*P*	$8{\cdot}4 \times 10^{-2}$	$1{\cdot}26 \times 10^{-2}$	0·15

Table 2. *Treatment of slices, and their absorption of phenosafranine*

(Slices with and without treatment were exposed for 60 min. in the glucose-saline medium with 10^{-5} M-phenosafranine, aerobically. Respiration was followed manometrically, and the phenosafranine of slice and solution was determined at the end of the experiment. Slice wt./ml. medium, 20–22 mg.)

Treatment	Ratio: $\frac{\text{Concn. phenosafranine in slice}}{\text{Concn. phenosafranine in medium}}$	Respiration (% control)
None	90	(100)
None: medium contains 0·1 M-NaF	33	30
Slice placed in ethanol for 5 min. and rinsed in saline before experiment	24	2
Slice in saline put in bath at 50° for 5 min. before experiment	24	40
Slice in saline put in bath at 60° for 5 min. before experiment	27	8

In marked contrast to these results were those obtained with nicotine and nicotinamide, in which the concentration of substance in the slices was only 0·2 of that in the medium. It must, however, be noted that this result concerns much higher concentrations of added compounds, and that the difference may not be specific for the compounds concerned. The concentrations chosen for study were those at which the different substances disturbed carbohydrate metabolism.

Effect of changed conditions on the entry of heterocyclic compounds to tissue slices

Table 1 showed that there is no large or consistent difference in the distribution of the compounds examined, when bicarbonate replaced phosphate as the main buffer.

It was, however, noticed that the compounds tended to leak from the slices if the experimental vessels were left without shaking or if the slices were removed from saline at the end of an experiment. Further experiments showed that the general metabolism of the tissue conditioned the concentration of added compounds in it. This is shown in Fig. 1 with respect to three compounds. Phenosafranine, neutral red, and ethyl red are absorbed less rapidly by the tissue anaerobically in the absence of substrate, than aerobically with glucose. The content of, for example, phenosafranine in the tissue is anaerobically about half that aerobically. This corresponds to a large difference in the ratios of substance in tissue/medium, under the two conditions.

Other circumstances which disturbed the metabolic activities of the slices also greatly affected their absorption of added substances (Table 2).

Nature of the compounds after assimilation by slices

No chemical change after assimilation was found in the majority of cases. In many experiments with phenosafranine, neutral red, diazine green, ethyl red, and nicotine, the data permitted comparison of the amount of substance added with that found in the slice and medium. Recovery was normally between 95 and 105 %. In addition, phenosafranine was examined in detail and the absorption spectrum of material recovered from slices was determined between 400 and 800 mμ. The only absorption observed was between 450 and 570 mμ. with a maximum at 515, and close correspondence over that range with the absorption of phenosafranine itself. Any association between phenosafranine and tissue constituents was therefore broken by the method of extraction used. This employed only sodium sulphate and chloroform at room temperature.

structures could be seen. Against the background of very uniform colour the nuclei of nerve cells appeared somewhat less coloured, and some endothelial cells around capillaries, more coloured. The granules at which neutral red and diazine green became concentrated were not differentially stained.

2-Aminophenazine methosulphate (10^{-4} M) was also evenly distributed.

Neutral red. General staining was observed, together with the localized staining of granules which probably corresponded with those described by others (cf. Covel & Scott, 1929; Cowdry, 1944) as being stained by neutral red by normal histological techniques (see also Cobb, Cohen & Ney, 1938).

Diazine green. General staining was observed, with nuclei and nerve fibres less coloured than other structures. Small intracellular granules were more deeply stained; these appeared to be mitochondria which are differentially stained by the related

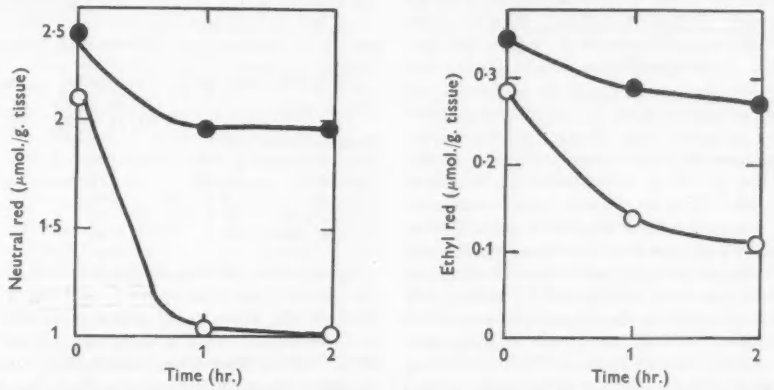

Fig. 2. Loss of neutral red and ethyl red from guinea pig-brain cortex slices. The substances were absorbed initially, aerobically, from glucose saline. Slices were then transferred: (*a*) to glucose saline and incubated aerobically, and (*b*) to saline without glucose, incubated anaerobically. The quantities of substances remaining in the slices are given above: (*a*) ●—●; (*b*) ○—○.

Histological localization of assimilated substances

Several of the substances examined are coloured. Portions of the guinea pig-brain cortex slices which had assimilated them during manometric experiments were examined promptly after mounting under a coverslip in the saline of the experiment, and the following observations made. The concentrations of dyestuffs examined were about those whose metabolic effects were under examination.

Phenosafranine. Guinea pig-brain cortex slices were examined after respiring in air for 0·5 and 2 hr. in glucose saline containing initially 10^{-5} M- and 5×10^{-5} M-phenosafranine (3 ml./slice of about 60 mg.). The general picture was of a uniformly pink tissue in which outlines of cells and intracellular

diazine green S (or Janus green B) after tissues have been fixed and dehydrated (cf. Cowdry, 1944).

Ethyl red. Staining was to a large extent general, and did not appear to be localized at any of the intracellular structures in the manner described above for other compounds. The first few outer layers of the cells of the cortex appeared more deeply stained.

Brilliant cresyl blue. This was localized in the fashion described for ethyl red, except that red blood cells when present were very strongly stained.

Loss of compounds from slices

In assessing the manner in which the added substances are held in the tissues, slices which had already assimilated them were transferred to fresh

media under various experimental conditions. The loss of added substances varied according to the presence or absence of glucose and oxygen (Fig. 2).

Actions of heterocyclic compounds on respiration and glycolysis

Choice of methods. The compounds examined included several which were absorbed by tissue slices over a period of an hour until a large proportion of added substance was within the slice. Comparison of the effects of different substances therefore required observations at frequent intervals such as those obtainable by manometric techniques. Determination of respiration in this manner was straightforward. In determining glycolysis, the applicability of the following considerations was examined. Gas exchange in a bicarbonate-buffered medium would be expected to be the summation of oxygen uptake, carbon dioxide formation and carbon dioxide displacement by acid. The respiratory quotient of brain cortex is almost exactly unity (Dickens, 1936*a*; cf. Elliott, Scott & Libet, 1942), so that determinations of respiration and total carbon dioxide evolution would enable the carbon dioxide displaced by acid to be found. Of methods for determining exchange in both carbon dioxide and oxygen, that employing two widely different volumes of bicarbonate-containing media presented the following difficulties. If, in determining the effect of a compound, equal concentrations of it were present in the fluid, with similar slices in the vessels, the two vessels would contain markedly different ratios of quantities of compound to tissue. As a large part of many of the compounds is finally concentrated in the slices, such procedure would give different final concentrations of the substances at their point of action. If, on the other hand, the ratio of the quantities of tissue and added substance is made equal in the two vessels, the concentration of dye in the tissues will be equal only if all added substance is absorbed by them. This was not the case. It was therefore decided to employ the method in which slices of similar weight are used in two vessels containing equal volumes of saline with the same concentrations of added compound. In one vessel respiration was measured in phosphate saline. In the other the net result of oxygen uptake and carbon dioxide output from bicarbonate saline was determined. Respiration in the two vessels was assumed to be equal and calculation of the carbon dioxide evolved followed Dixon (1943).

Experiments in which the applicability of the method was assessed are quoted in Table 3. In a series of experiments without added substances, lactic acid formation was determined in the bicarbonate-containing vessel colorimetrically, after periods of metabolism between 105 and 130 min. Manometric observations were begun in these experiments between 9 and 15 min. after mixing. Acid formation manometrically was then assessed by extrapolation to zero time of the rate observed between, for example, 10 and 120 min. The values obtained manometrically are lower than those observed chemically by a mean value of 13 %. This is considered to represent a reasonable agreement. The discrepancy is attributed to a small retention of carbon dioxide in the bicarbonate solution, and to the defect inherent in the method of allowing for the first few minutes' metabolism when no manometric observations could be made. This is known to extrapolate to a period of rapid lactic acid formation, the lower rate of the remaining experimental period (cf. Fig. 3 and Elliott *et al.* 1942).

Table 3. *Acid formation by slices; comparison of chemical and manometric methods*

Additions to media (M)		Products (μmol./g. wet wt.) determined after incubation (in different exp.) for 105–130 min. Lactic acid (colorimetrically)	CO_2 displaced (manometrically)
None (8 exp.)		40, 39, 52, 63 51, 44, 53, 49	43, 34, 40, 50 46, 42, 44, 40
Phenosafranine	10^{-5}	106	105
Phenosafranine	5×10^{-5}	126	142
Ethyl red	5×10^{-7}	111	102
Ethyl red	$1{\cdot}6 \times 10^{-6}$	140	157
Diazine green	$2{\cdot}5 \times 10^{-5}$	75	90
Thionine blue	10^{-4}	40	42

Comparison of the colorimetric and manometric determinations of lactic acid resulting from glycolysis in the presence of added compounds is also included in Table 3. The manometric results here are 6 % higher than the colorimetric ones. These experiments include many in which the total level of lactic acid formation is raised, so that the two factors to which the lowered manometric results were attributed above, would be expected to have less effect. Moreover, extrapolation is now from a period of higher to one of lower lactic acid formation.

Lactic acid formation and absorption of added compounds in bicarbonate and phosphate salines. In a series of experiments without added heterocyclic compounds, lactic acid was determined colorimetrically after metabolism in both the bicarbonate and phosphate salines. It was almost invariably found to be greater in the bicarbonate saline (Table 4). In eleven experiments in which the total quantity formed in phosphate saline lay between 25 and 55 μmol./g., that in bicarbonate was (mean value) 45 % higher. This difference was reduced in sixteen experiments with added substances, to a mean value of + 5 %. The total lactic acid formation in the experiments with added compounds was much greater.

Table 4. *Acid formation by slices in phosphate and bicarbonate salines*

(Experiments were aerobic, lasting 80–150 min., and with about 20 mg. tissue/ml.)

Additions to media (M)		Lactic acid (μmol./g.) In phosphate	In bicarbonate
None (11 exp.)		22, 24, 40, 35, 32, 41, 39, 40, 45, 45, 55	40, 39, 52, 63, 51, 54, 72, 44, 53, 77, 49
Phenosafranine	5×10^{-5}	126	126
Ethyl red	5×10^{-7}	84	101
Ethyl red	$1{\cdot}6 \times 10^{-6}$	117	128
Ethyl red	3×10^{-6}	117	140
Ethyl red	10^{-5}	143	163
Diazine green	$2{\cdot}5 \times 10^{-5}$	73	75
Diazine green	5×10^{-5}	60	62
Diazine green	$7{\cdot}5 \times 10^{-5}$	88	80
Diazine green	$1{\cdot}7 \times 10^{-4}$	50	34

Uptake of added compounds by the tissue was also determined in phosphate and bicarbonate salines. Any differences between the two values were small. Some comparative values are included in Table 1.

Respiration. The rate of respiration of guinea pig-brain cortex slices during the present experiments was 60–66 μmol./g. moist wt./hr., or 0·46–0·51 μmol./mg. dry wt./hr., and it remained at this value during the 2 hr. of typical experiments. The effects of added substances on respiratory rates are included in Table 5, which is discussed more fully below. It will be seen that many of the substances examined affected respiration, a common finding being a small acceleration at lower concentrations and inhibition at higher ones (compare Dickens, 1936*b*).

The course of aerobic glycolysis. Manometric results from a typical experiment with three concentrations of phenosafranine are shown in Fig. 3. Phenosafranine, as shown by Dickens (1936*c*), greatly increases glycolysis. The μmol. of lactic acid formed can become numerically about equal to the μmol. of oxygen absorbed in respiration. This implies an increase in the rate of glucose breakdown to four times its normal value. Glycolysis in Fig. 3, in the absence of added substance, has fallen after 30–40 min. to a steady rate of about 10 μmol./g. tissue/hr. In the presence of the two higher concentrations of phenosafranine, glycolysis was accelerated during the first 50 min., after which it remained steady. The lower concentration of phenosafranine showed a combination of these two effects. The period during which glycolysis is accelerating corresponds to the period during which phenosafranine is being absorbed by the slices (Fig. 1); after 50 min., steady concentrations had been reached. On the basis of this result, and of similar ones with other heterocyclic compounds, it was decided that the values during the second hour of metabolism would give the most suitable basis for comparison of metabolic effects. The concentrations of phenosafranine in these slices were therefore determined 2 hr. after commencing incubation.

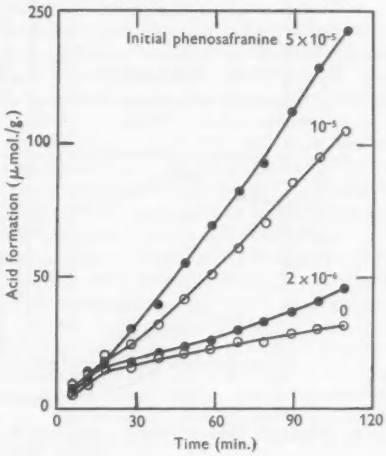

Fig. 3. Course of action of phenosafranine on aerobic glycolysis of guinea pig cerebral cortex slices, determined manometrically. Tissue weight: 25–28 mg./ml. Numbers by curves give the initial concentrations (M) of phenosafranine in the media.

The action of phenosafranine was then compared with the concentration in the slices, as shown in Fig. 4. Results with other compounds are also included in Fig. 4 and have been derived from other experiments of the type shown in Fig. 3. It is seen that all the substances under discussion, except possibly ethyl red, raise lactic acid formation at concentrations at which they do not inhibit, but may even accelerate, respiration. At the higher concentrations, inhibition of respiration is also observed in almost every case.

A given increase in rate of glycolysis is associated with the presence in the slices of widely different concentrations of the various substances. The numerical values for such concentrations, derived from Fig. 4, are the main outcome of this study and are discussed below in relation to other properties of the substances concerned.

Related compounds, and compounds with little or no effect on aerobic glycolysis. The substances in Fig. 4 are some only of the phenazine and pyridine derivatives whose action on glycolysis and respiration have been studied. Results with others are summarized in Table 5, where values for phenosafranine and nicotinamide are also quoted for comparison. The majority of the other compounds had much less action on aerobic glycolysis, and their concentrations in the slices were not determined.

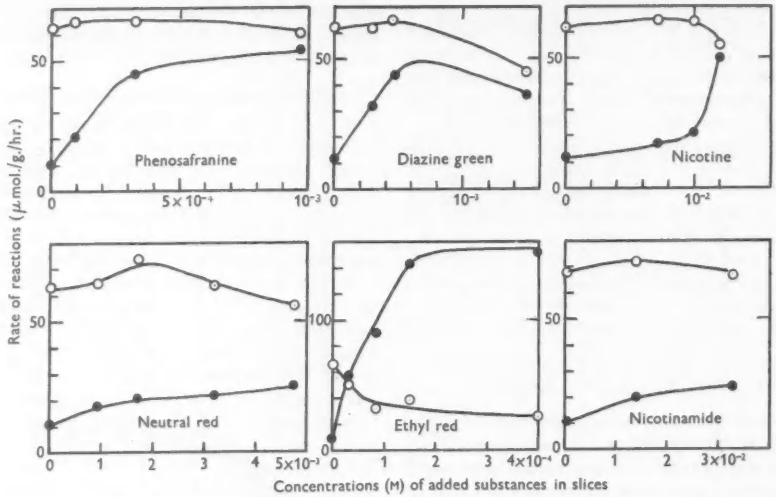

Fig. 4. Effects of added substances on respiration (○), and aerobic glycolysis (●), of guinea pig brain-cortex slices, during their second hour of metabolism.

Table 5. *Action of heterocyclic compounds on respiration and glycolysis of guinea pig-brain cortex*

(The values are derived from experiments of the type illustrated in Fig. 3.)

	Respiration (% of control)		Aerobic glycolysis (% of control)	
Substance (M)	1st hr.	2nd hr.	1st hr.	2nd hr.
Phenosafranine: 2×10^{-6}	105	103	117	230
10^{-5}	100	105	220	470
5×10^{-5}	100	78	310	690
2-Aminophenazine methosulphate:				
10^{-5}	139	133	73	90
10^{-4}	123	124	70	130
2×10^{-3}	124	42	110	60
Pyrocyanine: 10^{-3}	125	123	71	158
3×10^{-4}	129	128	55	90
Brilliant cresyl blue: 10^{-4}	155	100	100	105
10^{-3}	60	15	95	56
Thionine blue: 10^{-4}	130	70	92	105
10^{-3}	70	14	100	90
Nicotine: $3{\cdot}3 \times 10^{-2}$	100	105	128	145
$8{\cdot}3 \times 10^{-2}$	98	88	210	450
Pyridylglyoxaline: $3{\cdot}3 \times 10^{-3}$	94	90	156	275
Nicotinamide: 2×10^{-2}	98	95	108	150
5×10^{-2}	94	91	132	230
Nicotinamide methiodide: 10^{-2}	72	72	80	30
Trigonelline chloride: 10^{-2}	100	100	105	100
Coramine: 10^{-2}	89	89	100	130
Pyridine-3-sulphonamide: 10^{-2}	105	105	95	95
3-Acetylpyridine: 10^{-2}	71	71	80	50
10^{-3}	100	100	90	100
Quinine: 5×10^{-3}	5	5	78	15
10^{-3}	5	3	91	100

The magnitude of the difference can be seen by comparing the result with phenosafranine with those given by the aminophenazine methosulphate, pyocyanine, brilliant cresyl blue, or thionine blue. Only the first two of these showed any tendency to increase aerobic glycolysis, and this was relatively small. Our observations with pyocyanine agree with those of Young (1937). The effects of the pyridine derivatives are to be compared with that of nicotine and again are small except in the case of the pyridylglyoxaline whose action is noteworthy.

DISCUSSION

Absorption and exclusion by slices. The results emphasize how important it is in tissue-slice experiments to study the assimilation into the tissue of substances whose metabolic effects are under investigation. The phenazine derivatives reached concentrations in respiring brain-cortex slices, of the order of 100-fold those in the surrounding media. These concentrations were not reached only by a process of directly combining with tissue constituents, or staining the tissue, but were dependent on metabolic activities in the slice. Thus, the concentrations fell to about one-third of their value in the absence of glucose oxidation. The degree of concentration of these compounds in the tissue is thus an instance of the active transport of materials across cell barriers, which has been the subject of many studies in other systems (cf. Hoeber, 1945; Davson & Danielli, 1943). Some staining probably takes place also, as the tissue absorbs a certain

quantity of, for example, phenosafranine, when glucose is not being oxidized. Also when a slice which has assimilated phenosafranine aerobically is placed in anaerobic conditions without glucose, much but not all of the phenosafranine is released.

An implication of these findings in metabolic studies with slices is that a given concentration of phenosafranine in a medium could be expected to have different effects aerobically and anaerobically, on the basis only of the different concentrations reached in the slice. The substances studied showed a range of over 1000-fold in the ratio of their concentrations in tissue and in medium. The physiological significance of the systems which assimilate, e.g. phenosafranine to cortex slices is unknown, but they may represent the means by which heterocyclic compounds of importance to the tissue are assimilated from the blood stream. The uptake by the slices of ethyl red, neutral red and phenosafranine was initially at rates of some 0·3–1 μmol./g. moist wt./hr. (Fig. 1). In the case of phenosafranine, the tissue slices appeared to take up the compound uniformly, but the results with diazine green and neutral red raise questions of the distribution of the added substances intracellularly.

Action of glycolysis. Certain substances only, of those examined, increased aerobic glycolysis without marked inhibition of respiration. Among pyridine derivatives nicotine, nicotinamide, and the pyridylglyoxaline were active. These all have an unsubstituted pyridine N, and in the 3-position a substituent which includes the grouping R_1R_2C–NHR_3. The inactive compounds trigonelline chloride, coramine, pyridine-3-sulphonamide, and 3-acetylpyridine differ in one or more ways from this structure. The most active phenazine derivatives have basic substituents in positions 3 and 6, and have an *N*-phenyl group (for structures see McIlwain, 1950). Neutral red, without the *N*-phenyl group, is, however, active to some extent; the oxazine and thiazine, brilliant cresyl blue and thiazine blue, were inactive.

Comparison with the results of the preceding paper shows that the compounds which increase aerobic glycolysis are those which inhibit cozymase degradation, while those not increasing glycolysis have little or no action on cozymase degradation. A compound such as quinine as well as those named above, is included within this generalization.

The six compounds with largest effects on cozymase degradation and glycolysis were selected for a quantitative comparison of their effects on the two processes. These were the phenazine derivatives, phenosafranine, diazine green, neutral red; ethyl red, which is a quinoline derivative; and the pyridine derivatives nicotine and nicotinamide. The comparison is shown in Table 6. In this comparison, questions of the intracellular distribution of the different substances have not been taken into consideration. This appears justified because comparison has been between (i) the concentrations of substances added to suspensions of ground or homogenized brain in isotonic saline in which subcellular particles still exist as formed elements, and (ii) the amount of substance found to be reached within a given weight of the intact tissue, and expressed as an average concentration. Any selective absorption would be expected to be similar in the two cases, although an element of uncertainty remains.

In Table 6, columns A give the concentrations of the substances which are needed to raise aerobic glycolysis to arbitrarily chosen values. These concentrations were obtained from Fig. 4. The effects which these concentrations have on cozymase degradation were then found from concentration-action curves derived from data such as those of Tables 3, 5 and 6 of McIlwain (1950). These values are quoted in columns B, Table 6. There is a marked uniformity in the actions of four if not five of these compounds. Phenosafranine, diazine green, neutral red, and nicotine all raise aerobic glycolysis to 15 μmol./g./hr. in concentrations which inhibit cozymase degradation by 52–65 %; they raise it to 30 μmol./g./hr. in concentrations inhibiting degradation by 72–79 %. The actual concentrations of substances in the slices which are needed to produce a given effect on glycolysis or on cozymase differ in the different

Table 6. *Inhibition of cozymase degradation which is associated with given effects of different substances on aerobic glycolysis*

(Derivation of the values is described in the text.)

	Columns A, concentration (M) raising the rate of aerobic lactic acid formation to (μmol./g./hr.):			Columns B, inhibition of cozymase degradation caused by the concentration of substance which raises the rate of acid formation (col. A) to (μmol./g./hr):		
Added substance	15	30	60	15	30	60
Phenosafranine	6×10^{-5}	$1{\cdot}6 \times 10^{-4}$	7×10^{-4}	58	79	approx. 95
Diazine green	7×10^{-5}	$2{\cdot}4 \times 10^{-4}$	—	52	75	—
Neutral red	$1{\cdot}4 \times 10^{-3}$	$5{\cdot}5 \times 10^{-3}$	—	58	72	—
Ethyl red	10^{-5}	$1{\cdot}5 \times 10^{-5}$	3×10^{-5}	<5	<5	<5
Nicotinamide	5×10^{-3}	—	—	82	—	—
Nicotine	6×10^{-3}	$1{\cdot}1 \times 10^{-2}$	—	65	73	—

compounds by a factor of 100. The concentrations which are required of them in solution to produce comparable effects, differ by over 10,000-fold. The approximation of the values in columns B with respect to these four compounds is therefore noteworthy. In the case of nicotinamide, a given increase in glycolysis was found to be associated with a somewhat greater inhibition of cozymase degradation. The difference is not, however, great (and nicotinamide, unlike the other compounds, could conceivably yield cozymase). The result with ethyl red, however, is radically different from that with the other compounds. Inhibition of cozymase degradation is not apparent until a concentration is reached which is 10–30 times that at which glycolysis is affected. The substance thus belongs to those discussed earlier (McIlwain, 1950) which increase aerobic glycolysis without affecting cozymase degradation. The relation between cozymase degradation and the disturbance of glycolysis is being examined further.

SUMMARY

1. Slices of guinea pig-brain cortex shaken aerobically in glucose saline absorbed a number of phenazine and quinoline derivatives (initially at 10^{-7} to 10^{-4}M) until their concentrations in the slices were 50–300 times those in the solutions. Phenosafranine, diazine green, neutral red, and ethyl red were among the substances studied quantitatively. Nicotine, and nicotinamide, initially at 10^{-3} to 8×10^{-2}M, were largely excluded from the slices.

2. Absorption from solutions, with 20–30 mg. moist weight of slice/ml., took place gradually over a period of about an hour and at a maximum rate of about 1 μmol./g. moist wt./hr.

3. The absorption was greater aerobically with glucose than anaerobically without glucose; continued metabolism of glucose was necessary for the phenazine and quinoline derivatives to be held in the tissue to their maximum extent.

4. The substances named above have the common property of increasing aerobic glycolysis in brain-cortex slices, when present in concentrations which have little or no effect on respiration. The course of their action on aerobic glycolysis has been followed. Observations have been made on the structural requirements in such compounds, for increase of aerobic glycolysis, by examining a number of related substances.

5. The actions of many of the substances on carbohydrate metabolism have been evaluated in terms of the concentrations of substances actually present in the slices. The relative potencies so obtained for the compounds differed by up to 100-fold from their relative potencies expressed in terms of the concentrations of the compounds present in the initial reaction mixtures.

6. The concentrations of substances in the slices have been used as the basis for comparing the actions of the substances on carbohydrate and on cozymase metabolism.

We are greatly indebted to Prof. A. Meyer for assessing the histological localization of some of the compounds studied; and to Mr J. Cheshire for assistance during these experiments.

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Studies in Peroxidase Action

4. THE OXIDATION OF 4-METHOXY-2:6-DIMETHYLANILINE. SUGGESTIONS FOR A MECHANISM

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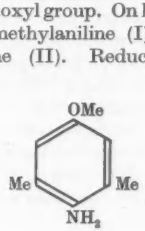
(*Received* 27 *September* 1949)

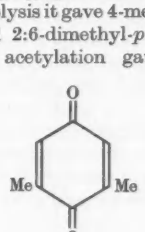
In Part 3 of this series (Chapman & Saunders, 1941) it was shown that mesidine (2:4:6-trimethylaniline) was oxidized at pH 4·5 by hydrogen peroxide and peroxidase, with the facile loss of a methyl group to a purple crystalline compound which proved to be 2:6-dimethylbenzoquinone-4(2′:4′:6′-trimethyl)-anil. The yield, using highly purified peroxidase, was 95%. It has now been shown manometrically that mesidine is not oxidized by indophenol oxidase, catechol oxidase or laccase (Keilin & Saunders, unpublished work).

In the reaction mentioned above the elimination of a methyl group at room temperature was unexpected. Attempts to ascertain the fate of the methyl group were not very conclusive. The main purpose of the present investigation was to examine the peroxidase oxidation of some related amine in which the *para* substituent was again likely to be eliminated, but was of such a nature that it might be possible to trace the products of fragmentation.

For this purpose we selected 4-methoxy-2:6-dimethylaniline, the synthesis of which is described later. Using the purified enzyme, and carrying out the oxidation at pH 4·5, a crystalline purple compound of m.p. 87–88° was obtained. The compound had an empirical formula $C_{17}H_{19}O_2N$ and contained a methoxyl group. On hydrolysis it gave 4-methoxy-2:6-dimethylaniline (I) and 2:6-dimethyl-*p*-benzoquinone (II). Reductive acetylation gave the



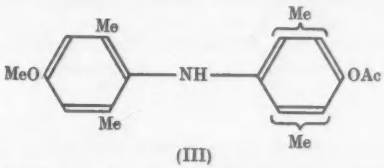
(I)



(II)

mono-acetyl derivative of a 4-*hydroxy*-4′-*methoxy*-*tetramethyl*-*diphenylamine* (III), the precise constitution of which is discussed below.

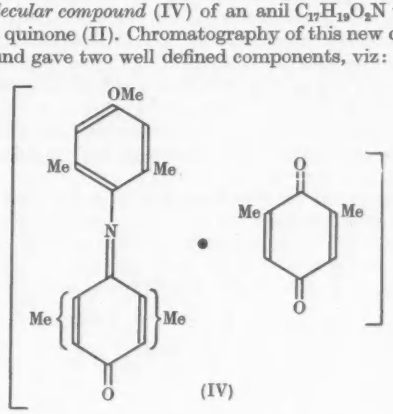
It seemed possible that the oxidation product might be synthesized from the hydrolysis products (I) and (II). Accordingly these two compounds were fused together in equimolecular quantities, a dark red product being obtained. This was recrystallized from aqueous ethanol as dark purple needles (darker than the actual oxidation product) of m.p. 36°. It was therefore not identical with the oxidation product. Analysis indicated that it was an *addition*



(III)

compound of 1 molecule of (I) and 1 molecule of (II). This was confirmed by the fact that it could be separated chromatographically into its components, and, moreover, the solution in light petroleum became progressively lighter in colour on dilution. This addition compound was probably of the 'quinhydrone' type.

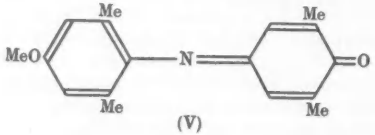
In a second experiment the amine (I) was dissolved in dilute acetic acid and the pH adjusted to 4·5. One molecular proportion of 2:6-dimethyl-*p*-benzoquinone dissolved in ethanol was then added slowly, and on standing, fine deep purple needles separated. When recrystallized from ethanol these had m.p. 78·5°. The analysis corresponded with a *molecular compound* (IV) of an anil $C_{17}H_{19}O_2N$ with the quinone (II). Chromatography of this new compound gave two well defined components, viz:



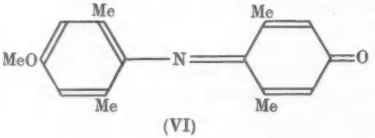
(IV)

2:6-dimethyl-*p*-benzoquinone and a compound identical in every respect with the oxidation pro-

duct from the peroxidase reaction. Furthermore, reductive acetylation of (IV) gave the substituted diphenylamine (III). These facts showed that the oxidation product was either 2:6-*dimethylbenzoquinone*-4-(4′-*methoxy*-2′:6′-*dimethyl*) *anil* (V) or 3:5-dimethyl-benzoquinone-4(4′-methoxy-2′:6′-dimethyl)anil (VI). At first sight, however, it



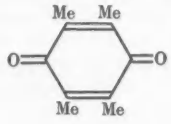
(V)



(VI)

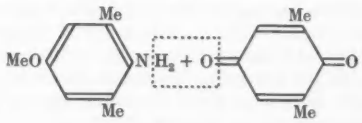
seemed puzzling that reductive acetylation of the original oxidation product and of (IV) gave only the monoacetyl derivative (III) and not the *ON*-diacetyl derivative. Steps were then taken to determine whether the monoacetyl derivative was the *O*- or the *N*-compound. When (III) was treated with concentrated sulphuric acid and a drop of very dilute nitric acid, a transient red coloration was obtained, which on standing changed to yellow. The production of a coloration with this oxidizing agent is consistent with the diphenylamine structure shown. Attempts to acetylate (III) further by acetic anhydride, acetic anhydride-acetic acid mixtures and acetyl chloride-pyridine failed. This resistance to acetylation led us to examine the acetylation of 4-methoxydiphenylamine itself. Using an acetic anhydride-acetic acid mixture, a red gum was obtained, but no crystalline acetyl derivative was isolated under the conditions used. The formation of a red colour indicated that a certain amount of oxidation was probably taking place. It is not therefore surprising that the reductive acetylation of the anil should tend to stop with the formation of the *O*-acetyl derivative.

The choice between formulae (V) and VI) for the oxidation product did not prove difficult for we found that 2:3:5:6-tetramethyl-*p*-benzoquinone (VII) would not condense with the amine (I) although it would form an addition compound.

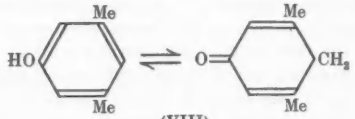


(VII)

Evidently the methyl groups cause steric hindrance. This suggests that formula (V) is correct and that its synthesis is effected as follows:



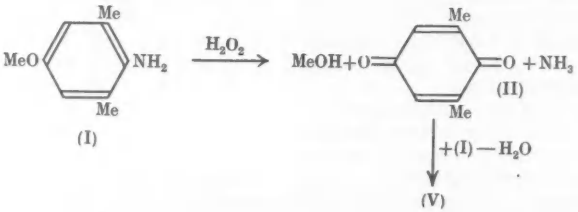
It is known that *p*-nitrosodimethylaniline will condense with phenol (acting in the tautomeric quinonoid form). We have shown that 3:5-dimethylphenol (VIII) will not condense with *p*-nitroso-



(VIII)

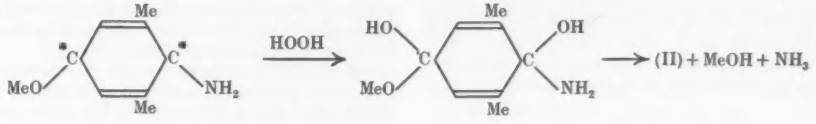
dimethylaniline, thus providing further evidence of the steric effect of the *o*-methyl groups, and hence giving additional support in favour of formula (V).

In the peroxidase oxidation of mesidine (Chapman & Saunders, 1941) a methyl group was lost, but the fate of the methyl group was obscure. In the present experiment with 4-methoxy-2:6-dimethylaniline, the filtrate after the removal of the solid oxidation product was made alkaline and treated as described in the Experimental section. Besides ammonia, the presence of methanol was established by the isolation of a small quantity of its 3:5-dinitrobenzoyl derivative. The formation of (V) may therefore be represented thus:



It should be emphasized that the first stages shown above represent only the overall oxidation of (I). If the primary product were the free radical $MeO.C_6H_2Me_2.NH\cdot$ we should expect the azo compound to be produced in good yield, but this compound was not produced in detectable quantity.

A very tentative explanation of this peroxidic conversion of (I) into (II) is now made as follows. It is suggested (cf. Saunders, 1949) that an intermediate (X) might be formed. This is both a hemiketal and a ketone-ammonia and such groupings are known to be very unstable, particularly in aqueous solution. The facile elimination of methanol and ammonia with the simultaneous production of (II) would therefore be the expected course of the reaction. The formation of (X) might be explained by assuming an initial transfer of two π electrons from (I) and their localization as p-electrons giving the diradical (IX). The contribution of (IX) to the real structure of 4-methoxy-2:6-dimethylaniline must be very small, and to achieve the conversion I→IX the resonance energy of the aromatic system must be supplied. The diradical may then react with the hydrogen peroxide giving (X). Such a reaction does not imply the presence of hydroxyl radicals in the system, but merely suggests that (IX) may cause a homolytic split of hydrogen peroxide into hydroxyl radicals (cf. Mackay & Melville, 1949) which then immediately react with (IX).



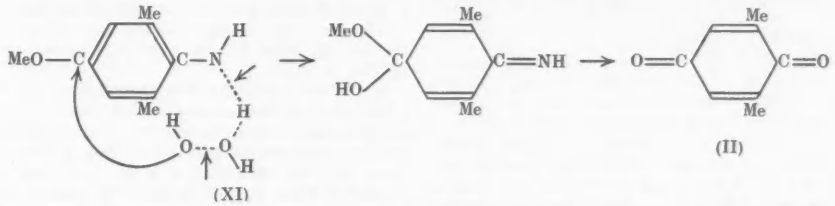
(IX) (X)

the production of a hydroxyl radical. A transition state may also be formulated with the hydrogen peroxide attached to the p-substituent.

Other alternative mechanisms for the conversion of (I) into (II) are clearly possible such as, for example, the initial removal of an electron from (I) giving (XII), which in the form (XIIa) would, after reaction with hydrogen peroxide, pass subsequently to (II).

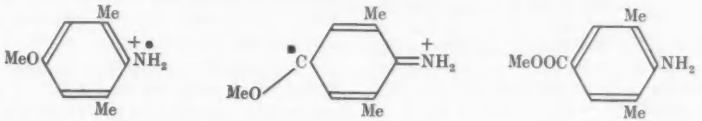
We would emphasize that the above suggestions are intended merely to serve as convenient working hypotheses for this type of oxidation. In this connexion, however, it is interesting to note that methyl 4-amino-3:5-dimethylbenzoate (XIII) failed to give a coloration when treated with hydrogen peroxide and peroxidase at pH 4·5. Such a compound containing a strong electron-attracting substituent might perhaps be less likely either (*a*) to react directly with hydrogen peroxide as above, or (*b*) to permit the removal of an electron for the suggested alternative mechanism. In fact the oxidizability of a compound by the peroxidase system may depend primarily upon the tendency to such electronic changes. p-Nitroaniline, for example, does not appear to be oxidized by peroxidase.

Any mechanism along the above lines for the oxidation of mesidine (Chapman & Saunders, 1941), would seem to require the loss of ammonia and methane in giving rise to the quinone. Chapman & Saunders obtained a quantitative yield of ammonia,



(XI)

It is not unlikely, however, that the reaction of (I) with hydrogen peroxide actually involves an activated complex such as (XI), but this again implies but were unable to decide the fate of the methyl group and did not detect formaldehyde which might result from a simple dehydrogenation mechanism.



(XII) (XIIa) (XIII)

To test the above suggestion, we propose to select as substrate a similar compound in which, however, the *p*-methyl group is replaced by a group which might give rise to a more readily detectable hydrocarbon.

Our substrate, 4-methoxy-2:6-dimethylaniline, had hitherto been prepared by laborious methods and accordingly we devised a new and more efficient synthesis as follows. 3:5-Dimethylphenol was coupled with benzenediazonium chloride and the phenolic hydroxyl group of the resulting azo compound methylated with dimethyl sulphate. The methoxyazo compound was then reduced with hydrogen and Raney nickel giving 4-methoxy-2:6-dimethylaniline.

An improved synthesis of methyl 4-amino-3:5-dimethylbenzoate was also effected by first oxidizing nitromesitylene with chromic anhydride in glacial acetic acid to 4-nitro-3:5-dimethylbenzoic acid. This on catalytic reduction gave the corresponding amino-acid, which was then esterified with methanol and hydrogen chloride giving methyl 4-amino-3:5-dimethylbenzoate (m.p. 104–106°). Meyer (1904) gave m.p. 93°. His analysis, however, was not good and so his specimen was probably impure.

EXPERIMENTAL

All m.p.'s are uncorrected.

4-*Hydroxy*-2:6-*dimethyl-azobenzene* (cf. Auwers & Michaelis, 1914). 3:5-Dimethylphenol (10 g.) was dissolved in aqueous NaOH (100 ml., 10%, w/v) and the solution cooled. Aniline (7·8 g.) was diazotized ($NaNO_2$ 6·5 g., 6N-HCl 30 ml.) and added slowly to the cold phenol solution, with stirring. The resulting mixture was allowed to stand at 5° for 30 min. and the solid product filtered off as a pale orange feathery mass (24·6 g.). This was digested with a mixture of 400 ml. of glacial acetic acid and 400 ml. of methanol, the mixture filtered and the filtrate poured into water (2·5 l.). On standing, 4-hydroxy-2:6-dimethyl-azobenzene separated and was recrystallized from light petroleum (b.p. 80–100°) as deep-red needles: m.p. 100–101°. Yield 12 g. (Found: N, 12·7. Calc. for $C_{14}H_{14}ON_2$: N, 12·4%.)

By this process 3:5-dimethylphenol-2:4-bisazobenzene (Auwers & Michaelis, 1914) was also produced, but this remained in solution when the reaction product was treated with 1:1-acetic acid-methanol mixture.

4-*Methoxy*-2:6-*dimethyl-azobenzene*. The foregoing monoazo-compound (6·4 g.) was shaken with dimethyl sulphate (4·5 g.) and NaOH (15 ml., 10% w/v) for 30 min. The product was filtered and recrystallized from aqueous ethanol as deep red plates: m.p. 53°. Yield 5·5 g. (Found: N, 11·8. Calc. for $C_{15}H_{16}ON_2$: N, 11·7%.)

4-*Methoxy*-2:6-*dimethylaniline*. 4-Methoxy-2:6-dimethylazobenzene (38 g.) was hydrogenated in ethanol at 100–110 atm. for 1 hr. using Raney Ni (30 g.) as catalyst. The product was filtered, the ethanol distilled off and the residue fractionated giving the amine of b.p. 135°/12 mm. This solidified to colourless plates which were recrystallized from light petroleum (b.p. 40–60°) and had m.p. 41°. (Found: N, 9·38. Calc. for $C_9H_{13}ON$: N, 9·42%.)

Oxidation of 4-*methoxy*-2:6-*dimethylaniline*. (*a*) *On a small scale*. The amine (0·41 g.) was dissolved in water (20 ml.) containing acetic acid (0·5 g.). The pH was about 4. A dilute solution of peroxidase (0·5 ml. of P.N. 100)* was added, followed by H_2O_2 (0·2 ml., 20 vol.). Further similar quantities of H_2O_2 were added every 20 min. and the enzyme (0·1 ml.) every 30 min. The following changes were noted. On the addition of H_2O_2 a reddish purple coloration was produced immediately. This gradually deepened and after about 30 min. purple solid began to separate. When the addition of H_2O_2 (3 ml.) was complete, the mixture was allowed to stand for 18 hr. and then filtered giving the crude crystalline product (0·255 g.). The 2:6-*dimethylbenzoquinone*-4-(4′-*methoxy*-2′:6′-*dimethyl*) *anil* was recrystallized from aqueous ethanol: m.p. 87–88°. (Found: C, 76·2; H, 6·85; N, 5·42; MeO, 10·9%. Mol.wt. cryoscopic in benzene, 259. $C_{17}H_{19}O_2N$ requires C, 75·8; H, 7·05; N, 5·2; MeO (one), 11·4%. Mol.wt. 269.)

(*b*) *On a larger scale*. The amine (6·8 g.) was dissolved in glacial acetic acid (8·2 ml.) and diluted with water (350 ml.). The whole was divided into 5 equal parts, and to each was added the enzyme solution (1 ml.). Hydrogen peroxide (1 ml.) was added every half hour until 6 ml. had been added (i.e. 30 ml. for the complete batch). Further small quantities of enzyme were also added at intervals. After standing for 18 hr., the solutions were combined and filtered giving the crude oxidation product (4·99 g.).

Hydrolysis of the oxidation product. The substance (1 g.) was heated under reflux with H_2SO_4 (60 ml., 10%, w/v), when a yellow sublimate collected on the cooler parts of the condenser. After 45 min. heating, the sublimate was washed out of the condenser with ethanol which, on concentration, gave 2:6-dimethyl-*p*-benzoquinone, m.p. and mixed m.p. with authentic specimen, 70·5°.

The acid solution was made alkaline with NaOH solution, extracted with ether, dried (Na_2SO_4), the ether distilled off and the residue fractionated giving pure 4-methoxy-2:6-dimethylaniline, b.p. 134°/12 mm.

Reductive acetylation of oxidation product. The compound (0·5 g.) was heated under reflux with acetic anhydride (60 ml.), pyridine (0·1 ml.), acetic acid (0·1 ml.) and zinc powder (about 0·5 g.). After 0·5 hr., the product was filtered and poured into ice water. On scratching, a solid separated which was recrystallized from aqueous methanol. 4′-*Acetoxy*-4-*methoxy*-2:6:3′:5′-*tetramethyldiphenylamine* was thus obtained as colourless needles, m.p. 127·5°. (Found: C, 73·0; H, 7·34; N, 4·7. $C_{19}H_{23}O_3N$ requires C, 72·9; H, 7·35; N, 4·63%.)

Fusion of 4-*methoxy*-2:6-*dimethylaniline and* 2:6-*dimethyl*-p-*benzoquinone*. The amine (0·5 g.) and the quinone (0·5 g.) were fused and allowed to cool. The product was recrystallized from aqueous ethanol as very dark purple glistening plates, m.p. 36°. The solution in light petroleum was red. (Found: C, 71·5; H, 7·17; N, 5·21. The *addition compound of the amine and quinone*, $C_9H_{13}ON$, C_8H_8O requires C, 71·4; H, 7·31; N, 4·89%.)

Synthesis of 2:6-*dimethyl-benzoquinone*-4-(4′-*methoxy*-2′:6′-*dimethyl*) *anil*. 4-Methoxy-2:6-dimethylaniline (0·224 g.) was dissolved in glacial acetic acid (0·2 ml.) and water (1 ml.) added. One drop of acetic acid was then added to clarify

* Purpurogallin number (P.N.) is defined as the number of mg. of purpurogallin formed by 1 mg. of peroxidase preparation, when allowed to act upon 5 g. of pyrogallol in 2 l. of water for 5 min. at 20° in presence of 10 ml. of 0·5% H_2O_2.

the solution. 2:6:Dimethyl-*p*-benzoquinone (0·2 g.) was dissolved in the minimum volume of ethanol and added drop by drop during a period of 30 min. (thus imitating the conditions of the peroxidic oxidation of the amine). A purple coloration resulted, but no solid separated. After standing, however, for 48 hr. in a refrigerator, violet needles separated and were recrystallized from aqueous ethanol, m.p. 78·5°. (Found: C, 74·1; H, 6·85; N, 4·11. The *addition compound of the anil and quinone*, $C_{17}H_{19}O_2N$, $C_8H_9O_2$ requires C, 74·0; H, 6·77; N, 3·5%.)

This addition compound was dissolved in light petroleum (b.p. 80–100°) and examined chromatographically on alumina. Two bands were obtained: (A) pale yellow; (B) deep purple. (A) travelled quickly through the alumina and proved to be 2:6-dimethyl-*p*-benzoquinone. Elution of (B) with ethanol followed by concentration gave a purple solid which, on recrystallization from aqueous ethanol, gave purple crystals of m.p. 87–88°. Mixed m.p. with peroxidase oxidation product, 87·5°. (Found: C, 75·7; H, 7·1; N, 5·3. Calc. for $C_{17}H_{19}O_2N$: C, 75·8; H, 7·05; N, 5·2%.)

Attempted condensation of p*-nitrosodimethylaniline with 3:5-dimethylphenol.* (i) The phenol (1·7 g.) was dissolved in NaOH solution (20 ml., 10%, w/v), and powdered *p*-nitrosodimethylaniline (1·5 g.) and Zn dust (3 g.) were added. The mixture was shaken for 2 hr. and allowed to stand for 24 hr. A dark blue coloration developed and the product was shaken with ether. On dilution with more ether, the blue coloration disappeared indicating that a molecular compound had been formed. On concentrating the ether and cooling, crystals of unchanged *p*-nitrosodimethylaniline were obtained.

(ii) The phenol and *p*-nitrosodimethylaniline were heated together in xylene solution, under reflux, for 2 hr. A dark tarry material was obtained which appeared to contain decomposition products of the nitroso compound.

Examination of the filtrate from the oxidation product. The filtrate was allowed to stand for 7 days to complete the oxidation, and then filtered. A small quantity of a crystalline purple compound was obtained. It had m.p. 79° and was proved to be the addition compound of the anil and the quinone.

The filtrate was made alkaline and filtered free from traces of a red amorphous material. It was then fractionated using a Fenski column and 2·5 ml. of a liquid of b.p. 69–78° were obtained. On shaking this liquid with powdered 3:5-dinitrobenzoyl chloride and NaOH solution, a solid was obtained which was recrystallized from light petroleum. It had m.p. 184° and was proved, by mixed m.p. and by its general reactions, to be 3:5-dinitrobenzamide thus showing that NH_3 was a product of the peroxidase reaction. The mother liquor from the amide was concentrated, and colourless needles were obtained which, after four recrystallizations, had m.p. 103° and the mixed m.p. with authentic methyl 3:5-dinitrobenzoate was 103°. Thus the presence of methanol in the original filtrate was proved.

4-Nitro-3:5-dimethylbenzoic acid. Nitromesitylene (10 g.) was dissolved in glacial acetic acid (25 ml.) and heated in a boiling water bath. CrO_3 (7 g.) in glacial acetic acid (100 ml.) and water (20 ml.) was slowly added during 1 hr. and the mixture stirred for a further 5 hr. The product was poured into ice water, made alkaline with NaOH solution and steam distilled. The residual liquid was acidified with HCl and cooled, when the acid separated. It was recrystallized as colourless prisms from aqueous ethanol and had m.p. 215°. Yield, 1·6 g. (Found: C, 55·6; H, 4·94; N, 7·45. Calc. for $C_9H_9O_4N$: C, 55·4; H, 4·67; N, 7·62%.)

Methyl 4-amino-3:5-dimethylbenzoate. The foregoing nitro acid (1·6 g.) was dissolved in ethanol (75 ml.) and a small quantity of Adams's PtO_2 catalyst added. The mixture was hydrogenated at 75 atm. and 100° for 1 hr., then filtered and evaporated to dryness *in vacuo* yielding the crude amino-acid (5 g.). (This could be recrystallized from ethanol in the form of long needles, m.p. 248°, decomp.). The crude acid was dissolved in methanol and heated under reflux for 2 hr. with a stream of dry HCl passing through. The solution was concentrated, poured into water, neutralized with NaOH solution, and the amino-ester separated after standing for 2 hr. It was recrystallized from aqueous methanol and had m.p. 104–106°. Yield 3·2 g. Meyer (1904) claimed that the ester had m.p. 93°. (Found: C, 66·48; H, 6·48; N, 7·62. Calc. for $C_{10}H_{13}O_2N$: C, 67·0; H, 6·53; N, 7·83%.)

Attempted oxidation of methyl 4-amino-3:5-dimethylbenzoate. Considerable difficulty was encountered because of the low solubility of the compound in dilute acid, but a series of experiments was carried out on a test-tube scale at pH 4–6 and at concentrations of about 0·01–0·05%. The addition of peroxidase and H_2O_2 produced no coloration, and the amino-ester was recovered unchanged in each case.

SUMMARY

1. Peroxidase and hydrogen peroxide oxidize 4-methoxy-2:6-dimethylaniline to a crystalline purple compound which by degradation and unambiguous synthesis has been proved to be 2:6 - dimethylbenzoquinone - 4(4′ - methoxy - 2′:6′ - dimethyl) anil. Ammonia and methanol are also produced during the oxidation.

2. Hypothetical intermediate compounds have been postulated to explain the reaction.

3. It appears that amines containing powerful electron-attracting groups are not readily oxidized by the peroxidase system.

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Biochemical Studies of Toxic Agents

1. EXPERIMENTS WITH RADIOACTIVE 2:3-DIMERCAPTOPROPANOL (BRITISH ANTI-LEWISITE)

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During the period 1941–5, studies were conducted in this laboratory on the antidotal action of 2:3-dimercaptopropanol (BAL, British Anti-Lewisite) and other thiols in arsenic poisoning; for review see Young (1946). The investigations were undertaken after receiving early reports of the work of Peters and his co-workers on the value of 2:3-dimercaptopropanol as an antidote to arsenic. In 1945 Peters, Stocken & Thompson reviewed investigations on BAL which had been carried out at Oxford and elsewhere during the War, and, since the appearance of their article, numerous papers describing researches on BAL have been published.

The fact that no satisfactory method for determining BAL in biological material was available led us to synthesize BAL with radioactive sulphur (^{35}S) incorporated in the molecule (Simpson & Young, 1945*a*) and to use it in studies of the fate of BAL in the animal body (Simpson & Young, 1945*b*,*c*). A series of experiments was carried out on rats in which the absorption of BAL by the skin was allowed to proceed for a period of 6 hr. after application and the unabsorbed BAL was then removed. The absorption, distribution and excretion of ^{35}S were determined at 6, 12 and 24 hr. after the initial application (Simpson & Young, 1945*b*). A second series of experiments was conducted in which the distribution and excretion of ^{35}S were determined at 6, 12 and 24 hr. after the intramuscular injection into the rat of a solution of radioactive BAL in propylene glycol (Simpson & Young, 1945*c*).

An account of similar work with radioactive BAL, with results in general agreement with those described herein, has been published by Peters, Spray, Stocken, Collie, Grace & Wheatley (1947). These authors indicate that their work with radioactive BAL was in progress when reports describing some of the present investigations appeared.

EXPERIMENTAL

Analytical methods

The determination of S and the measurement of ^{35}S were carried out by the procedures described by Young, Edson & McCarter (1949) in which the material is oxidized by the Carius method and the sulphate is precipitated as benzidine sulphate.

'SH' was determined by titration of a solution of the compound either with standard I_2 solution or with standard KIO_3 solution in the presence of HCl and excess KI (Simpson, Zbarsky & Young, 1944).

Synthesis of radioactive BAL

Dithiols are often prepared by the interaction of sodium hydrosulphide or ammonium hydrosulphide and the appropriate dihalide under pressure (cf. Simpson, 1947). The method can be used for the synthesis of 2:3-dimercaptopropanol with radioactive S incorporated in the molecule if H_2S containing $H_2{}^{35}S$ is used to prepare the hydrosulphide. Radioactive BAL was prepared in the present work by allowing radioactive sodium hydrosulphide to react with 2:3-dibromopropanol in methanol solution.

Radioactive mustard gas was the only source of ^{35}S immediately available to us, and all that could be spared for the synthesis of radioactive BAL was 20 mg. This amount of radioactive mustard gas was converted to H_2S with negligible loss of ^{35}S by a modification of ter Meulen's method for determining S in organic compounds (ter Meulen, 1922; Masters, 1939). The mustard gas was heated in a stream of H_2 and the vapour passed over strongly heated platinized asbestos. The H_2S formed was collected in 0·5 ml. of aqueous NaOH solution. In five preliminary experiments, in each of which the H_2S formed by the decomposition of 20 mg. of non-radioactive mustard gas was collected in this manner, the recoveries of S (as determined iodometrically) varied from 97 to 101% (mean, 98%) of the theoretical value. The Na_2S was converted by the action of H_2S to sodium hydrosulphide, and this was used in the preparation of BAL. Several preparations of non-radioactive BAL were carried out in order to determine suitable conditions for the reaction between 2:3-dibromopropanol and sodium hydrosulphide. The results led to the use of methanol as solvent for the reagents in the presence of the least possible amount of water, and not less than four molecules of sodium hydrosulphide for each molecule of 2:3-dibromopropanol.

Procedure. Radioactive mustard gas (10 mg.) and an equal amount of non-radioactive mustard gas were placed in a platinum boat lined with filter paper. The boat was then introduced into a Vycor combustion tube (Corning Glass Works), which contained loosely packed platinized asbestos. The method of preparing the combustion train and the procedure used in the combustion were similar to those described by Masters (1939) for the determination of S in organic compounds and biological material. Pure H_2 was passed through the combustion tube for 5 min. to displace air. The platinized asbestos was then heated strongly and the portion of the combustion tube containing the platinum

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boat was heated gently so that the mustard gas vaporized slowly and was carried through the platinized asbestos by the H_2. The issuing gases were bubbled through a solution of 0·15 g. of NaOH dissolved in 0·5 ml. of water contained in a 15 ml. tapered centrifuge tube. The whole process was repeated with another 10 mg. sample of radioactive mustard gas and the H_2S was collected in another 0·5 ml. of NaOH solution. The two centrifuge tubes containing the radioactive Na_2S were set aside in an ice bath until required for the next stage of the synthesis.

Methanol (15 ml.) was placed in a 200 ml. glass pressure flask and 0·977 g. of metallic Na was added. The flask was placed in an ice bath and H_2S was bubbled through the methanol solution for 4 hr. The contents of the two centrifuge tubes were then transferred to the pressure flask. Each centrifuge tube was washed with a total of 5 ml. of cold methanol in small portions and these washings were added to the contents of the pressure flask. The flask was kept chilled in ice and H_2S was passed under pressure into the solution for 6 hr.

At the end of this period 0·0115 g.mol. of 2:3-dibromopropanol (Eastman Kodak Co.) was chilled and added to the radioactive sodium hydrosulphide (0·050 g.mol.) in the pressure flask. The flask was then sealed with a rubber stopper which was wired in position. The flask was kept at 30° for 80 hr. and was then chilled and opened. Concentrated HCl (2·4 ml.) was added slowly until the contents of the flask were acid to congo red. (The gas which was liberated during this process contained $H_2{}^{35}S$ and was collected as CdS.) The pressure flask and its contents were heated in a water bath at 50° and the methanol was then removed by evaporation under reduced pressure. The flask and its contents were cooled to room temperature and 10 ml. of water were added in order to dissolve the solid material in the flask. The contents of the flask were then extracted with five 10 ml. portions of peroxide-free ether, and the combined extracts were dried over anhydrous Na_2SO_4. The ether was evaporated and a residue which weighed 1·629 g. was obtained. This material was distilled under reduced pressure and the fraction which boiled at 66° at 0·025 mm. pressure was collected. The weight of this fraction was 0·663 g. and represented a yield of BAL of 46% based on the amount of dihalide used. (Found: 'SH' (by iodine titration), 51·8; S, 50·7%. Calc. for $C_3H_8OS_2$: 'SH', 53·2; S, 51·6%.) The specific activity of the radioactive BAL was 64% of the maximum specific activity possible under the experimental conditions used.

RESULTS

Distribution of ^{35}S following the application of radioactive BAL to the skin of the rat

A study was made of the distribution of ^{35}S in the rat and its excreta at 6, 12 and 24 hr. after the application of radioactive BAL to the skin. Three experiments were performed in each of which 0·05 ml. of radioactive BAL was applied to the clipped skin of the back of a rat. In each experiment the site of application was covered with a glass and celluloid cap sealed to the back of the animal, and loss of BAL by evaporation and mechanical removal was thereby prevented. Absorption of BAL was allowed to proceed for 6 hr., and at the end of this period the BAL which remained on the skin was removed and its ^{35}S content was determined. After a known interval (0, 6 or 18 hr. after removal of the unabsorbed BAL, i.e. 6, 12 or 24 hr. after dosing) the rat was bled to death, dissected and analysed for ^{35}S. The urine and faeces were collected separately during the whole experimental period and were analysed for ^{35}S.

Procedure. Three male white rats (wt. 167, 157 and 173 g.) were used. The experiments were numbered as follows: rat no. 1, analysed at the end of the period of absorption of BAL (i.e. 6 hr. after dosing); rat no. 2, analysed 6 hr. after the end of the absorption period (i.e. 12 hr. after dosing); rat no. 3, analysed 18 hr. after the end of the absorption period (i.e. 24 hr. after dosing).

The hair in the central area of the back of each rat was clipped to the skin level by means of electric shears and scissors. A ring of glass with an inside diameter of 3·5 cm. and a depth of 1·5 cm. was sealed on to the clipped skin by means of glass cement (Duco Cement, Canadian Industries Ltd.). The animal was anaesthetized with ether and 0·05 ml. of radioactive BAL (63 mg.) was measured by means of a micropipette (Young, 1941, 1947) on to the skin within the ring. The BAL was rubbed evenly over the skin within the glass ring for 5 min. with the flattened end of a glass rod. The site of dosing was then closed by sealing a circular piece of celluloid, 4·5 cm. in diameter, over the top of the glass ring. The BAL which remained on the glass rod was washed into a 25 ml. volumetric flask with ethanol and the solution was made to volume. In order to determine the BAL content of the solution, 10 ml. portions were diluted with water, acidified with HCl and titrated with 0·02 N-KIO_3 solution in the presence of excess KI (Simpson *et al.* 1944). Hereafter the amount of BAL measured on to the skin minus the amount which adhered to the glass rod is described as 'radioactive BAL applied'.

After the animal had been dosed it was placed in a metabolism cage of a type which permitted the collection of urine separate from the faeces. Water was available to the rat while it was in the metabolism cage, but food was not provided. 6 hr. after the application of the BAL the rat was anaesthetized with ether in the metabolism cage (in these and other experiments the rat usually emptied its bladder during its struggles while being anaesthetized). The urine excreted during the 6 hr. period was analysed for ^{35}S. While the animal was still anaesthetized, the disc of celluloid covering the glass ring was removed and the dosed skin was wiped with three small pieces of cotton wool. The skin was next rubbed lightly with three pieces of cotton wool moistened with ethanol and after each rubbing it was wiped with dry cotton wool. All the pieces of cotton wool were dropped into ethanol and the mixture was stirred. The ethanol solution of the material removed from the skin was then washed into a 50 ml. volumetric flask through a funnel loosely plugged with cotton wool. The solution was made to volume and sample portions were analysed for their ^{35}S content. In the experiments in which the distribution of ^{35}S was studied at 6 and 18 hr. after the end of the absorption period, the celluloid disc was cemented over the glass ring after the BAL remaining on the skin had been removed. The rat was then returned to the metabolism cage. The dose of BAL applied minus the amount of BAL (based on the ^{35}S data) removed from the skin at the end

of the absorption period and that present on the cap and in the glass cement at the end of the experimental period is described hereafter as 'radioactive BAL absorbed'.

At the end of the experimental period each rat was anaesthetized in the metabolism cage and given an intravenous injection of 0·1 ml. of heparin solution (Connaught Laboratories, Toronto; 1000 units/ml.). It was then bled as completely as possible from the carotid artery. The blood was collected, its volume was measured, and 1 ml. samples were analysed for ^{35}S. The glass ring and the cement holding it to the back of the animal were removed and the amount of ^{35}S on them was determined. All the skin which had been covered by the cap was excised, weighed and analysed for ^{35}S. In order to avoid the possible transfer of ^{35}S from the dosed area to other parts of the animal, the dissecting instruments which were used in the above operation were set aside and others were used for the remaining dissection. Tissues removed from the animal were placed in stoppered glass weighing bottles, weighed, and duplicate samples were analysed for ^{35}S. The 'carcass' (i.e. all of the animal which remained after removal of the tissues which were analysed for ^{35}S) was weighed and its content of ^{35}S was measured.

Urine was collected and analysed for ^{35}S at the following times after dosing: rat no. 1, 6 hr.; rat no. 2, 6 and 12 hr.; rat no. 3, 6, 12 and 24 hr. The faeces were collected at the end of the experimental period and their ^{35}S content was measured.

The data in Table 1 shows the amounts of radioactive BAL applied and the amounts absorbed in the 6 hr. during which absorption took place. They also show the amounts of ^{35}S in the skin at the site of dosing, in the body of the rat and in the excreta at the end of the experimental period.

Table 1. *The distribution of* ^{35}S *at* 6, 12 *and* 24 *hr. after the application of radioactive BAL to the skin of the rat**

Rat no. ...	1	2	3
Time of killing after application of radioactive BAL (hr.)	6	12	24
Radioactive BAL applied (mg.)	62	58	60
Radioactive BAL absorbed in 6 hr. (mg.)	25	21	21
	% of ^{35}S applied as BAL		
^{35}S not absorbed in 6 hr.	60	64	65
^{35}S in skin at site of dosing	3	3	1
^{35}S in body of rat (excluding skin at site of dosing)	10	3	3
^{35}S in urine and faeces	14	22	25
Total ^{35}S traced	87	92	94

* Absorption of radioactive BAL by the skin was allowed to proceed for 6 hr.

The rate of absorption of BAL by the skin of the rat was low. In the three experiments just described, the amounts of BAL absorbed in 6 hr. were 25, 21 and 21 mg. In each experiment the area of dosed skin was 9·6 sq.cm. The rates of absorption in terms of BAL/sq.cm. of skin/hr. were therefore 0·43, 0·36 and 0·36 mg., and the mean value was 0·38 mg.

The fact that BAL or substances containing sulphur derived from BAL did not accumulate in the dosed skin to any marked extent is shown by the relatively small amounts of ^{35}S in the skin at the end of the 6 hr. period of absorption. For example, in the case of rat no. 1, which was killed at the end of the absorption period, the amount of ^{35}S found in the skin corresponded to 1·9 mg. of BAL (i.e. 7·6 % of the BAL absorbed) and was distributed in 5·84 g. of skin.

In the blood and most of the tissues of rat no. 1, ^{35}S was present in low concentration. The concentrations of ^{35}S were equivalent to the following amounts of BAL (mg.)/g. of fresh tissue: blood, 0·07; liver, 0·07; kidney, 0·22; heart, 0·04; lung, 0·07; spleen, 0·06; brain, 0·03; stomach, 0·04; small intestine and contents, 0·12; testis, 0·03; muscle under dosed skin, 0·03; leg muscle, 0·03; skin remote from dosed area, 0·05; and carcass, 0·03. Only in the kidney and in the small intestine and its contents were the concentrations of ^{35}S appreciably higher than in the other tissues apart from the skin at the site of dosing, and this was probably related to the passage of radioactive material into the urine and the bile.

As a result of excretion of ^{35}S in the urine, the amount of ^{35}S in the organism decreased rapidly after the end of the period of absorption of radioactive BAL. At 6 hr. after the end of the absorption period the liver did not contain measurable amounts of ^{35}S, and the concentration of ^{35}S in the kidney had fallen to the equivalent of 0·04 mg. BAL/g. of fresh tissue. At 18 hr. after the end of the absorption period neither the kidney nor the liver contained a measurable amount of ^{35}S.

A striking feature of the data is the rate at which ^{35}S was excreted in the urine (see also Table 3). This is discussed later. Small amounts of ^{35}S were found in the faeces, but these may have originated from urine which came in contact with the faeces.

Distribution of ^{35}S *following the intramuscular injection of BAL into the rat*

A study, similar in many respects to that just described, was made with rats which were given radioactive BAL by intramuscular injection. The distribution of ^{35}S was studied at 6, 12 and 24 hr. after injection. In each of the three experiments performed, 20 mg. of radioactive BAL dissolved in propylene glycol were injected into the muscle of the left hind leg of a rat. The animal was then placed in a metabolism cage and at the end of the experimental period it was killed, dissected and analysed for ^{35}S. The urine and faeces were also collected and analysed for ^{35}S.

Procedure. Three male white rats, each of which weighed 185 g., were used in the experiments. The experiments were numbered as follows: rat no. 4 was analysed at 6 hr., rat no. 5 at 12 hr. and rat no. 6 at 24 hr. after the injection of radioactive BAL.

The hair on the left hind leg of each rat was clipped to the skin level. The animal was anaesthetized with ether and a freshly prepared solution of 20 mg. of radioactive BAL in 0·30 ml. of propylene glycol was injected into the muscle of the left hind leg. The point of withdrawal of the needle from the skin was clamped for 10 min. and was then sealed with glass cement.

The animal was then placed in a metabolism cage and was provided with water but not with food. At the end of the experimental period the rat was anaesthetized with ether in the metabolism cage and was given an intravenous injection of 0·1 ml. of heparin solution. It was then bled as completely as possible from the carotid artery and 1 ml. samples of blood were analysed for ^{35}S. The muscle of the left hind leg ('dosed muscle') was removed and analysed for ^{35}S. The remainder of the experiment was conducted in the manner described for those experiments in which rats were dosed by application of radioactive BAL to the skin.

The main findings are shown in Table 2.

Table 2. *The distribution of* ^{35}S *at* 6, 12 *and* 24 *hr. after the intramuscular injection of a solution of radioactive BAL in propylene glycol into the rat*

Rat no.	4	5	6
Time of killing after injection of radioactive BAL (hr.)	6	12	24
Radioactive BAL injected (mg.)	20	20	20
	% of ^{35}S injected as BAL		
^{35}S in dosed muscle	2	5	6
^{35}S in body of rat (excluding dosed muscle)	33	12	11
^{35}S in urine and faeces	48	69	82
Total ^{35}S traced	83	86	99

The fact that BAL or substances containing sulphur derived from BAL were not retained to any great extent at the site of injection is shown by the relatively small amounts of ^{35}S which were found in the dosed muscle. The maximum amount of ^{35}S found in the dosed muscle corresponded to 1·2 mg. of BAL (i.e. 6 % of the dose) and this was present in 3·18 g. of muscle. Although the amount of ^{35}S in the dosed muscle appeared to increase with time, this may well have been due to slight differences in the rate of movement of the ^{35}S from the muscles of the different animals.

^{35}S was present in low concentration in the blood and a number of tissues 6 hr. after the injection of radioactive BAL (rat no. 4). The concentrations of ^{35}S were equivalent to the following amounts of BAL (mg.)/g. of fresh tissue: blood, 0·01; liver, 0·03; kidney, 0·06; heart, 0·04; lung, 0·03; spleen, 0·03; small intestine and contents, 0·12; skin, 0·03; carcass, 0·3. As in the case of rat no. 1, which was analysed 6 hr. after the application of radioactive BAL to the skin, the ^{35}S concentration of the small intestine and its contents was well above the average for the other tissues. In the kidney, however, the concentration of ^{35}S was not much higher than the average, and in this respect rat no. 4 differed from rat no. 1.

At 12 hr. after injection, but not at 24 hr. after injection, detectable amounts of ^{35}S were found in the blood, liver and kidney. ^{35}S was excreted quite rapidly in the urine of the injected animals (see also Table 3). The amounts of ^{35}S found in the faeces were very small and may have originated from contamination of the faeces with urine.

Urinary excretion of ^{35}S *after the administration of radioactive BAL to the rat*

Table 3 shows the amounts of ^{35}S excreted in the urine in various periods after the administration of radioactive BAL to the rat by application to the skin or by intramuscular injection. The amounts of radioactive BAL which were absorbed when the compound was left in contact with the skin for 6 hr. (25, 21 and 21 mg.) were not greatly different from the amount administered (20 mg.) in each of the experiments in which the compound was injected intramuscularly, and in both sets of experiments the rate of excretion of ^{35}S was approximately the same. This rate of excretion was such that the ^{35}S from about three-quarters of the dose of radioactive B was excreted in the 24 hr. following the administration of the compound.

Table 3. *The urinary excretion of* ^{35}S *by the rat in* 0–6, 0–12 *and* 0–24 *hr. following the administration of radioactive BAL by application to the skin* or by intramuscular injection*

Rat no.	1	2	3	4	5	6
Radioactive BAL absorbed by the skin (mg.)	25	21	21	—	—	—
Radioactive BAL injected intramuscularly (mg.)	—	—	—	20	20	20
Urine collection period	% of ^{35}S absorbed as BAL			% of ^{35}S injected as BAL		
0–6 hr. after administration	35	43	43	47	50	46
0–12 hr. after administration	—	61	65	—	66	71
0–24 hr. after administration	—	—	72	—	—	81

* Absorption of radioactive BAL by the skin was allowed to proceed for 6 hr.

Some interest attaches to the form in which ^{35}S is excreted in the urine of animals dosed with radioactive BAL. As a preliminary step in a study of this question the stability of BAL dissolved in urine was investigated. Aqueous solutions of BAL were prepared and added to normal rat urine so as to yield solutions which contained 1·0 mg. and 0·1 mg. of BAL/ml. After measured intervals of time, sample portions of these solutions were titrated with standard potassium iodate solution in the presence of hydrochloric acid and excess potassium iodide. A similar set of experiments was performed using

rat urine which had been made just acid to congo red by the addition of hydrochloric acid. The results obtained are given in Table 4, and they show that the thiol content of the solutions of BAL in urine decreased rapidly, but that this process occurred more slowly in urine which had been acidified.

Table 4. *The thiol content of normal rat urine and acidified rat urine at various times following the addition of BAL*

	Initial concn. of BAL in urine			
	1·0 mg./ml.		0·1 mg./ml.	
	Percentage of initial thiol concn.*			
Time (min.)	Normal urine	Acidified urine	Normal urine	Acidified urine
5	95	100	—	100
30	75	99	52	97
(hr.)				
1	63	98	43	95
3	22	96	20	85
6	10	93	6	73
12	4	88	—	60
24	—	76	—	33

* All values are corrected for the 'blank' thiol concentration of the urine.

An experiment was performed in which a male white rat (wt., 180 g.) was given an intramuscular injection of 20 mg. of radioactive BAL in 0·30 ml. of propylene glycol under the conditions already described. The animal was placed in a metabolism cage and its urine was collected in a flask which contained sufficient 2N-hydrochloric acid to render the urine just acid to congo red. The animal was anaesthetized in the metabolism cage at the end of 24 hr. after dosing and this caused it to empty its bladder. The total ^{35}S content of the urine was determined by the method described by Young *et al.* (1949). The ^{35}S content of the total sulphate fraction of the urine was determined as follows.

Urine (5 ml.) to which 5 ml. of 3N-HCl had been added was evaporated to dryness by heating on a water bath. Water (3 ml.) was added to the residue, and this yielded an acid solution containing a small amount of insoluble material. The mixture was centrifuged, the supernatant fluid was removed and the residue was washed with three 1 ml. portions of water and centrifuged after each washing. The supernatant fluid and washings (total vol., 6 ml.) were combined, and 4 ml. of 95% (v/v) ethanol and 4 ml. of benzidine hydrochloride reagent were added. The benzidine sulphate which formed was separated and its ^{35}S content was measured. The insoluble residue obtained after the hydrolysis of the urine was transferred to a Carius bomb, and its ^{35}S content was determined by the method of Young *et al.* (1949).

The amounts of ^{35}S (expressed as percentages of the ^{35}S injected as radioactive BAL) found in the various fractions of the urine were as follows: total sulphur fraction, 74; total sulphate fraction, 3; insoluble fraction obtained after hydrolysis, 8%. Under the experimental conditions used, it appears unlikely that the insoluble fraction contained significant amounts of ^{35}S in the form of sulphate. Even if all the ^{35}S in the insoluble fraction was in the form of sulphate, 63% of the ^{35}S administered as BAL was present in the neutral sulphur fraction of the urine. It can be stated, therefore, that a large proportion (63–71%) of the ^{35}S injected as BAL was excreted in 24 hr. in the neutral sulphur fraction of the urine, and only a small proportion (3–11%) was excreted in the form of sulphate in this period. These findings are similar to those obtained by Peters *et al.* (1947) following the administration of a propylene glycol solution of radioactive BAL to rats by intraperitoneal, subcutaneous or intramuscular injection.

The presence of a large proportion of the ^{35}S in the neutral sulphur fraction of the urine made it appear possible that unchanged BAL was excreted in the urine.

In order to investigate this possibility three male rats (weighing 178, 180 and 185 g.) were each given an intramuscular injection of 20 mg. of radioactive BAL dissolved in 0·30 ml. of propylene glycol. The animals were placed in a metabolism cage over a glass funnel and the urine was collected for 6 hr. in a vessel which contained sufficient 2N-HCl to render the urine acid to congo red. Analysis of a sample of the urine showed that 46% of the ^{35}S administered as BAL was excreted during this period. At the end of the collection period an aqueous solution (5 ml.) of 0·20 g. of non-radioactive BAL was added to the urine. The urine was then shaken with several portions of peroxide-free ether. The ether extracts were combined and dried over anhydrous Na_2SO_4. The ether was evaporated and the residue was distilled at low pressure. This yielded 0·172 g. of BAL; 'SH' content (by I_2 titration), 53·2%; calc. for $C_3H_8OS_2$, 53·2%. This material was converted to 2-phenyl-4-hydroxymethyl-1:3-dithiolane by refluxing it on a water bath for 6 hr. with 10 ml. of benzene, 0·28 ml. of benzaldehyde and three drops of conc. HCl. The mixture was then evaporated to dryness in a current of air. The residue was dissolved in 1 ml. of benzene and precipitated by the addition of 2 ml. of light petroleum (b.p. 30–60°). This process was repeated and the crystalline material obtained was then extracted with boiling *n*-hexane. The *n*-hexane was removed by evaporation and the residue was recrystallized three times from benzene-light petroleum mixture. The dithiolane thus obtained weighed 0·200 g.; m.p. 66–68°. (Found: C, 56·4; H, 5·6; S, 30·5%. Calc. for $C_{10}H_{12}OS_2$: C, 56·6; H, 5·7; S, 30·2%.) When this compound was analysed for ^{35}S it was found that its radioactivity was too low to measure.

The conditions in the experiment just described were such that if 1% of the administered radioactive BAL had been present in the unchanged form at the end of the collection period and had then been converted to the dithiolane, this derivative would have contained measurable amounts of ^{35}S. The fact that this experiment yielded no evidence for the presence

of unchanged BAL in the urine does not rule out the possibility that the unchanged compound was excreted. While it seems unlikely that a major portion of the ^{35}S was excreted in the form of BAL it should be noted that the thiol content of a solution of BAL in urine decreases on standing even when the urine has been acidified, and small amounts of BAL, especially if they were excreted in the early part of the experimental period, might well have undergone chemical change by the end of the period during which urine was collected.

DISCUSSION

The present investigation has shown that BAL is absorbed slowly by the skin of the rat, for in the 6 hr. period following the application of radioactive BAL to the skin the average rate of absorption of ^{35}S corresponded to 0·38 mg. BAL/sq.cm. skin/hr. Values similar to this were obtained in experiments in which non-radioactive BAL was applied to the skin of the rat and the amount of unabsorbed BAL was determined by iodate titration (Simpson *et al.* 1944). It seems probable that the rate of absorption of BAL by human skin does not differ greatly from that found for the skin of the rat. In experiments in which radioactive BAL was applied to the flexor surfaces of the forearms of two human subjects and evaporation from the dosed areas was prevented by the method used in the experiments with rats, the average rate of absorption of ^{35}S during the 3 hr. period following dosing corresponded to 0·59 and 0·24 mg. BAL/sq.cm. skin/hr. The low rate of absorption of BAL is particularly interesting in view of the fact that the direct application of BAL to skin contaminated with lewisite is highly effective in preventing injury to the skin. Whether the rate of absorption of BAL by the skin is altered in the presence of lewisite has not been determined.

When the purpose of BAL adminstration is to combat systemic arsenical poisoning (or poisoning by other heavy metals for which BAL has been shown to be an antidote) it would appear desirable to administer the BAL by a method which makes it available within the organism more rapidly than would occur if it were applied to the skin. The mode of administration of BAL commonly employed for this purpose is intramuscular injection and the present work has shown that absorption of radioactive material takes place readily from muscle which has been injected with a solution of radioactive BAL in propylene glycol.

6 hr. after the administration of radioactive BAL to the rat by application to the skin or by intramuscular injection ^{35}S was found to be present in many tissues. It is not known, however, whether this ^{35}S was present as BAL or in the form of compounds incapable of exerting an antidotal action. Even if all the ^{35}S in the organism under these conditions was in the form of BAL (and this seems unlikely) the total amount present was considerably less than that which had been absorbed from the skin or injected intramuscularly. The main reason for the falling off in the amount of ^{35}S in the organism was the passage of radioactive material into the urine, e.g. in the 6 and 12 hr. periods following the intramuscular injection of radioactive BAL 46–50 and 66–71% respectively of the ^{35}S administered were excreted in the urine.

Most of the ^{35}S excreted in the 24 hr. period following the intramuscular injection of radioactive BAL into rats was present in the neutral sulphur fraction of the urine and relatively little was present in the total sulphate fraction. The nature of the ^{35}S-containing compound (or compounds) in the neutral sulphur fraction of the urine has yet to be established. Stocken & Thompson (1946) reported that the subcutaneous or intraperitoneal injection of BAL into rats or rabbits was followed by the excretion of an unidentified thiol (or thiols) in the urine, and Spray, Stocken & Thompson (1947) found that a considerable proportion of the thiol(s) present in urine obtained from catheterized rabbits after the intramuscular injection of BAL appeared to be present as a compound closely related to BAL, but not identical with it. In the present work no evidence was obtained for the presence of unchanged radioactive BAL in the urine of rats dosed with this compound. A negative finding under the conditions employed is not conclusive, however, for it is possible that some unchanged BAL was excreted and then underwent chemical change while the urine was being collected. It may be that BAL is excreted in the urine in conjugation with some other substance. This possibility has received some support from the work of Spray (1947), who found that there was an increased excretion of glucuronic acid in the urine of rats in the 24 hr. period following the injection of BAL.

SUMMARY

1. A description is given of the synthesis of 2:3-dimercaptopropanol (BAL) with radioactive sulphur (^{35}S) incorporated in the molecule.

2. The absorption, distribution and excretion of ^{35}S have been studied following the administration of radioactive BAL to rats by application to the skin or by intramuscular injection.

3. When radioactive BAL is applied to the skin of the rat it is absorbed slowly, the ^{35}S which is absorbed is distributed throughout the organism and most of it is excreted in the urine within the following 24 hr.

4. When radioactive BAL in propylene glycol is administered to rats by intramuscular injection, the ^{35}S passes almost completely from the dosed muscle in 6 hr. Radioactive material is present in most of

the tissues 6 hr. after the injection. A large proportion of the ^{35}S is excreted in the neutral sulphur fraction of the urine in the 24 hr. following the injection of radioactive BAL.

A short account of this work was presented at a meeting of the Biochemical Society held at St Thomas's Hospital Medical School, London, S.E. 1, on 18 February 1949 (Simpson & Young, 1949).

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